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(54) Title: NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF
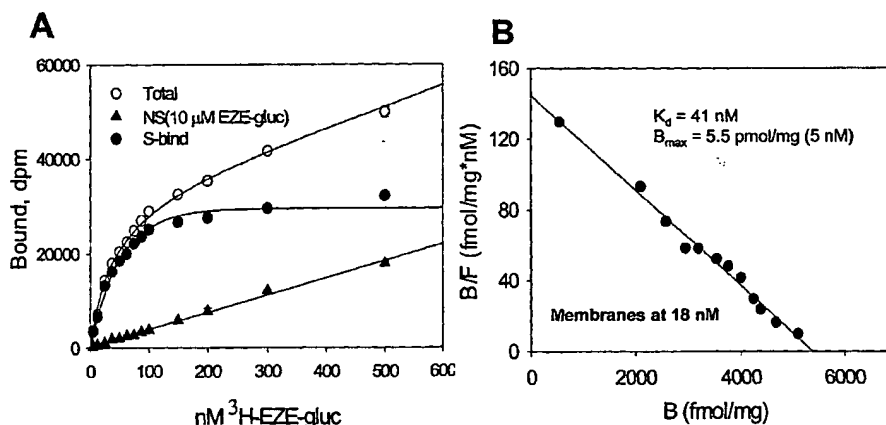
Equilibrium binding of EZE-glucuronide to rhesus BBMVs

(57) Abstract: The present invention provides human, rat and mouse NPC1L1 polypeptides and polynucleotides encoding the polypeptides. Methods for detecting ligands which bind to NPC1L1 and block intestinal cholesterol absorption are provided. Also included is a method of identifying ligands which bind to NPC1LI using membranes derived from brush border membrane preparations. Compounds that bind to NPC1LI can be used for inhibiting intestinal cholesterol absorption in a subject.

NPC1L1 (NPC3) AND METHODS OF IDENTIFYING

LIGANDS THEREOF

SPECIFICATION

5   The invention claimed herein was made on behalf of Merck & Co.,

Inc. and Schering-Plough Corporation, parties to a joint research agreement that was

in effect on or before the date the claimed invention was made.

          This application claims priority to Serial No. 60/537,341, filed

January 16, 2004.

10   FIELD OF THE INVENTION

          The present invention includes NPC1L1 polypeptides and

polynucleotides which encode the polypeptides, methods of use and methods of

identifying modulators and ligands thereof.

BACKGROUND OF THE INVENTION

15   A factor leading to development of vascular disease, a leading cause of

death in industrialized nations, is elevated serum cholesterol. It is estimated that 19%

of Americans between the ages of 20 and 74 years of age have high serum cholesterol.

The most prevalent form of vascular disease is arteriosclerosis, a condition associated

with the thickening and hardening of the arterial wall. Arteriosclerosis of the large

20   vessels is referred to as atherosclerosis. Atherosclerosis is the predominant

underlying factor in vascular disorders such as coronary artery disease, aortic

aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

          Cholesteryl esters are a major component of atherosclerotic lesions and

the major storage form of cholesterol in arterial wall cells. Formation of cholesteryl

25   esters is also a step in the intestinal absorption of dietary cholesterol. Thus, inhibition

of cholesteryl ester formation and reduction of serum cholesterol can inhibit the

progression of atherosclerotic lesion formation, decrease the accumulation of

cholesteryl esters in the arterial wall, and block the intestinal absorption of dietary

cholesterol.

The regulation of whole-body cholesterol homeostasis in mammals and animals involves the regulation of intestinal cholesterol absorption, cellular cholesterol trafficking, dietary cholesterol and modulation of cholesterol biosynthesis, bile acid biosynthesis, steroid biosynthesis and the catabolism of the cholesterol-

5   containing plasma lipoproteins. Regulation of intestinal cholesterol absorption has proven to be an effective means by which to regulate serum cholesterol levels. For example, a cholesterol absorption inhibitor, ezetimibe (
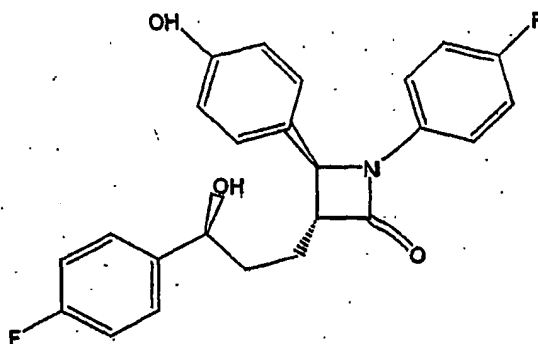
), has been shown to be effective in this regard. A pharmaceutical composition

10   containing ezetimibe is commercially available from Merck/Schering-Plough Pharmaceuticals, Inc. under the trade name Zetia®. Identification of a gene target through which ezetimibe acts is important to understanding the process of cholesterol absorption and to the development of other, novel absorption inhibitors. The present invention addresses this need by providing a rat and a mouse homologue of human

15   NPC1L1 (also known as NPC3; Genbank Accession No. AF192522; Davies, *et al.*, (2000) Genomics 65(2): 137-45 and Ioannou, (2000) Mol. Genet. Metab.71(1-2): 175-81), an ezetimibe target.

NPC1L1 is an N-glycosylated protein comprising a YQRL (SEQ ID NO: 38) motif (i.e., a *trans*-golgi network to plasma membrane transport signal; see

20   Bos, *et al.*, (1993) EMBO J. 12: 2219-2228; Humphrey, *et al.*, (1993) J. Cell. Biol. 120: 1123-1135; Ponnambalam, *et al.*, (1994) J. Cell. Biol. 125: 253-268 and Rothman, *et al.*, (1996) Science 272: 227-234) which exhibits limited tissue distribution and gastrointestinal abundance. Also, the human *NPC1L1* promoter includes a Sterol Regulated Element Binding Protein 1 (SREBP1) binding consensus

25   sequence (Athanikar, *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95: 4935-4940; Ericsson, *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 945-950; Metherall, *et al.*, (1989) J. Biol. Chem. 264: 15634-15641; Smith, *et al.*, (1990) J. Biol. Chem. 265:

2306-2310; Bennett, *et al.*, (1999) J. Biol. Chem. 274: 13025-13032 and Brown, *et al.*, (1997) Cell 89: 331-340). NPC1L1 has 42% amino acid sequence homology to human NPC1 (Genbank Accession No. AF002020), a receptor responsible for Niemann-Pick C1 disease (Carstea, *et al.*, (1997) Science 277: 228-231). Niemann-

5   Pick C1 disease is a rare genetic disorder in humans which results in accumulation of low density lipoprotein (LDL)-derived unesterified cholesterol in lysosomes (Pentchev, *et al.*, (1994) Biochim. Biophys. Acta. 1225: 235-243 and Vanier, *et al.*, (1991) Biochim. Biophys. Acta. 1096: 328-337). In addition, cholesterol accumulates in the *trans*-golgi network of npc1⁻ cells, and relocation of cholesterol, to and from the

10   plasma membrane, is delayed. NPC1 and NPC1L1 each possess 13 transmembrane spanning segments as well as a sterol-sensing domain (SSD). Several other proteins, including HMG-CoA Reductase (HMG-R), Patched (PTC) and Sterol Regulatory Element Binding Protein Cleavage-Activation Protein (SCAP), include an SSD which is involved in sensing cholesterol levels possibly by a mechanism which involves

15   direct cholesterol binding (Gil, *et al.*, (1985) Cell 41: 249-258; Kumagai, *et al.*, (1995) J. Biol. Chem. 270: 19107-19113; Hua, *et al.*, (1996) Cell 87: 415-426; and Radhakrishnan, A., *et al.*, "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)).

20   SUMMARY OF THE INVENTION

        The present invention is based on the discovery that NPC1L1 is the target through which ezetimibe acts, and consequently plays a critical role in the regulation of sterol and 5α-stanol intestinal transport and absorption, *e.g.* cholesterol absorption. Accordingly, this invention provides for the use of NPC1L1 in an assay

25   for identifying ligands that block NPC1L1-mediated sterol and 5α-stanol intestinal transport. The present invention provides methods for identifying ligands of NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1. The

30   modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of detectably labeled substituted 2-azetidinone to NPC1L1 in the presence

5   and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

The present invention also provides for a method for identifying a

10   compound that inhibits intestinal sterol or 5α-stanol absorption mediated by NPC1L1 involving contacting NPC1L1 with a detectably labeled ligand and the candidate compound and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1, wherein said modulation indicates that the candidate compound is

15   an intestinal sterol or 5α-stanol absorption inhibitor.

The present invention provides methods for identifying an ligand of NPC1L1 comprising (a) contacting a host cell (e.g., human embryonic kidney (HEK) 293 cells, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID

20   NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of a detectably labeled (e.g., with ^{3}H, ^{14}C, ^{125}I, ^{35}S or fluorescence labeling) substituted azetidinone (e.g., ezetimibe), with a sample to be tested for the presence of an NPC1L1 ligand; and (b) measuring the amount of detectably labeled substituted azetidinone (e.g., ezetimibe) specifically

25   bound to the polypeptide; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced binding of the detectably labeled substituted azetidinone (e.g., ezetimibe) to the polypeptide, compared to what would be measured in the absence of such a ligand.

Another method for identifying an ligand of NPC1L1 is also provided.

30   The method comprises (a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer (e.g., yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene), to which a host cell (e.g., chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a

polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface are attached; (b) adding, to the suspension, a radiolabeled (e.g., with ^{3}H, ^{14}C or ^{125}I) substituted azetidinone (e.g., ezetimibe) and a sample to be tested for the presence of a ligand,

5   wherein the radiolabel emits radiation energy capable of activating the fluorescer upon the binding of the substituted azetidinone (e.g., ezetimibe) to the polypeptide to produce light energy, whereas radiolabeled substituted azetidinone (e.g., ezetimibe) that does not bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and (c) measuring

10   the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such a ligand.

Also provided is a method for identifying a ligand of NPC1L1 comprising (a) contacting a host cell (e.g., Chinese hamster ovary (CHO) cell, a J774

15   cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface with detectably labeled (e.g., with ^{3}H, ^{14}C or ^{125}I) sterol (e.g., cholesterol) or 5α-stanol and with a sample to be tested for the presence of an ligand; and (b) measuring the amount of detectably labeled sterol (e.g.,

20   cholesterol) or 5α-stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol (e.g., cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an antagonist and wherein an NPC1L1 agonist in the sample is identified by measuring substantially increased detectably labeled sterol (e.g.,

25   cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an agonist.

The present invention includes methods for inhibiting NPC1L1-mediated intestinal sterol (e.g., cholesterol) or 5α-stanol uptake, in a subject, by administering a substance identified by the screening methods described herein to the

30   subject. Such substances include compounds such as small molecule antagonists of NPC1L1 other than ezetimibe. Also contemplated are methods for antagonizing NPC1L1-mediated sterol (e.g., cholesterol) or 5α-stanol absorption by administering anti-NPC1L1 antibodies. NPC1L1-mediated absorption of sterol (e.g., cholesterol) or

5α-stanol can also be antagonized by any method which reduces expression of
NPC1L1 in an organism. For example, NPC1L1 expression can be reduced by
introduction of anti-sense *NPC1L1* mRNA into a cell of an organism or by genetic
mutation of the *NPC1L1* gene in an organism (*e.g.*, by complete knockout, disruption,
5   truncation or by introduction of one or more point mutations).

Also included in the present invention is a mutant transgenic mammal
(*e.g.*, mouse, rat, dog, rabbit, pig, guinea pig, cat, horse), preferably a mouse
comprising a homozygous or heterozygous mutation (*e.g.*, disruption, truncation, one
or more point mutations, knock out) of endogenous, chromosomal *NPC1L1* wherein,
10   preferably, the mouse does not produce any functional NPC1L1 protein. Preferably,
the mutant mouse, lacking functional NPC1L1, exhibits a reduced level of intestinal
sterol (*e.g.*, cholesterol) or 5α-stanol absorption and/or a reduced level of serum sterol
(*e.g.*, cholesterol) or 5α-stanol and/or a reduced level of liver sterol (*e.g.*, cholesterol)
or 5α-stanol as compared to that of a non-mutant mouse comprising functional
15   NPC1L1. Preferably, in the mutant mouse chromosome, the region of *NPC1L1* (SEQ
ID NO: 45) deleted is from nucleotide 790 to nucleotide 998. In one embodiment,
NPC1L1 (SEQ ID NO: 11) is deleted from nucleotide 767 to nucleotide 975. Any
offspring or progeny of a parent *NPC1L1* mutant mouse (i.e., *npc1l1*) of the invention
which has inherited an *npc1l1* mutant allele is also part of the present invention.

20   The scope of the present invention also includes a method for
screening a sample for an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption
antagonist comprising (a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing
substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-
cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to
25   a third, mutant mouse lacking a functional *NPC1L1*; (b) administering the sample to
the first mouse comprising a functional *NPC1L1* but not to the second mouse; (c)
measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the
intestine of said first, second and third mouse (*e.g.*, by measuring serum cholesterol);
and (d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol
30   absorption in each mouse; wherein the sample is determined to contain the intestinal
sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal
sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and third mouse are

less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the second mouse.

The present invention also encompasses a kit comprising (a) a substituted azetidinone (*e.g.*, ezetimibe) in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 10 mg substituted azetidinone (*e.g.*, ezetimibe)); and (b) information, for example in the form of an insert, indicating that NPC1L1 is a target of ezetimibe. The kit may also include simvastatin in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 5 mg, 10 mg, 20 mg, 40 mg or 80 mg simvastatin). The simvastatin in pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form can be associated in a single pill or tablet or in separate pills or tablets.

The present invention also provides any isolated mammalian cell (*e.g.*, isolated mouse cell, isolated rat cell or isolated human cell) which lacks a gene which encodes or can produce a functional NPC1L1 polypeptide. The isolated cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein. Further, the mutation can be in a gene which when un-mutated encodes an amino acid sequence of SEQ ID NO: 12 (*e.g.*, comprising a nucleotide sequence of SEQ ID NO: 11). The cell can be isolated or derived from duodenum, gall bladder, liver, small intestine or stomach tissue. The cell can be an enterocyte.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide to rhesus brush border membrane (BBM) vesicles. Observed total binding (Total) is shown as open circles; nonspecific binding (NS) as triangles, and specific binding (S-bind) as solid circles.

Figure 1B shows a scatchard analysis of ^{3}H-EZE-glucuronide binding to rhesus brush border membrane vesicles.

Figure 2A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide (1) to rat brush border membrane vesicles. Observed total binding (open circles) and nonspecific binding (triangles), determined in the presence of 100 μM unlabeled ezetimibe glucuronide, are included; specific binding (solid circles) was assessed from the difference between total and nonspecific binding.

Binding was measured at 2.5 mg protein/ml in a volume of 100 μl after 1 hour

incubation. Data were fit by nonlinear regression as described in Methods.

Figure 2B shows scatchard analysis of ^{3}H-EZE-glucuronide binding to

rat brush border membrane vesicles. The binding data identify a single high-affinity

5   site with K_D = 542 nM and Bmax = 20.7 pmol/mg protein.

Figure 3A shows association kinetic analysis of ^{3}H-EZE-glucuronide

in rat brush border membrane vesicles. Conditions were 25 nM of $\underline{1}$ and 3 mg/ml

protein at 25°C. The second-order rate constant k_{on} (0.55×10^{-4} M^{-1} s^{-1}) was

calculated from k_{obs} (0.004 s^{-1}) as described in Methods.

10   Figure 3B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide

$\underline{1}$ in rat brush border membrane vesicles. After the complex was formed by

incubating 25 nM of $\underline{1}$ and 3 mg/ml protein for 1 hour, dissociation was initiated by

competition with 100 μM unlabeled ezetimibe glucuronide. The curve is theoretical

for k_{off} = 0.0024 s^{-1}.

15   Figure 4A shows association kinetic analysis of ^{3}H-EZE-glucuronide

in rhesus brush border membrane vesicles.

Figure 4B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide

in rhesus brush border membrane vesicles.

Figure 5 shows the results of a binding assay where ^{3}H-EZE-

20   glucuronide is dissociated by EZE-glucuronide and compound $\underline{2}$ from rhesus (A) and

rat (B) brush border membrane vesicles.

Figure 6 shows the results of a binding assay where ^{35}S-$\underline{2}$ is dissociated

by EZE-glucuronide and $\underline{2}$ from mouse brush border membrane vesicles.

Figure 7 shows the distribution of ^{3}H-EZE-glucuronide binding to

25   rhesus (A) and rat (B) brush border membranes prepared from various portions of

rhesus (A) and rat (B) intestinal tissue.

Figure 8 shows the results of a binding assay where ^{35}S-$\underline{2}$ is dissociated

by EZE-glucuronide and various analogs from CHO cells transfected with rat

NPC1L1.

30   Figure 9 shows the results of a binding assay where ^{35}S-$\underline{2}$ is dissociated

by EZE-glucuronide and various analogs from CHO cells transfected with human

NPC1L1.

Figure 10 shows the binding of ^{35}S-$\underline{2}$ to brush border membrane vesicles prepared from wild type (A) and *NPC1L1* knockout (-/-) mice.

Figure 11 shows the results of a binding assay where ^{35}S-$\underline{2}$ is dissociated by compound $\underline{2}$ from mouse wild type and *NPC1L1* knockout (-/-) brush border membrane vesicles.

Figure 12A shows equilibrium determination of K_D for ezetimibe glucuronide by competition of unlabeled compound against $\underline{1}$ in rat enterocyte brush border membranes. Membranes (1.5 mg/ml protein) were incubated with $\underline{1}$ (50 nM) and the indicated concentrations of ezetimibe glucuronide for 1 hour to ensure equilibrium. K_D at equilibrium is 600 nM. Figure 12B shows the corresponding measurement for rhesus monkey, which were conducted between 0.5 and 1.25 mg/ml protein and 22-50 nM $\underline{1}$, with incubation time of more than 3 hours. K_D at equilibrium is 38.6 nM.

Figure 13 shows the expression of NPC1L1 in HEK-293 cells using Western blot analysis (Panel 1) and immunofluorescence (Panel 2).

Figure 14A shows binding of ^{3}H-ezetimibe glucuronide to enterocyte brush border membranes from wild type mice and NPC1L1 deficient mice in the presence of detergent. Figure 14B shows competition studies of unlabeled ezetimibe glucuronide against labeled ezetimibe glucuronide.

Figure 15 shows the effect of detergents, taurocholate and digitonin, on [^{3}H]ezetimibe glucuronide binding.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes NPC1L1 polypeptides from rat, human and mouse, along with polynucleotides encoding the respective polypeptides. Preferably, the rat NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2, the human NPC1L1 comprises the amino acid sequence set forth in SEQ ID NO: 4 and the mouse NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 12. The rat *NPC1L1* polynucleotide of SEQ ID NO: 1 or 10 encodes the rat NPC1L1 polypeptide. The human *NPC1L1* polynucleotide of SEQ ID NO: 3 encodes the human NPC1L1 polypeptide. The mouse *NPC1L1* polynucleotide of SEQ ID NO: 11 or 13 encodes the mouse NPC1L1 polypeptide.

The present invention includes any isolated polynucleotide or isolated polypeptide comprising a nucleotide or amino acid sequence referred to, below, in Table 1.

Table 1. Polynucleotides and Polypeptides of the Invention.

Polynucleotide or Polypeptide	Sequence Identifier
Rat *NPC1L1* polynucleotide	SEQ ID NO: 1
Rat *NPC1L1* polypeptide	SEQ ID NO: 2
Human *NPC1L1* polynucleotide	SEQ ID NO: 3
Human *NPC1L1* polypeptide	SEQ ID NO: 4
Rat *NPC1L1* expressed sequence tag 603662080F1 (partial sequence)	SEQ ID NO: 5
Rat *NPC1L1* expressed sequence tag 603665037F1 (partial sequence)	SEQ ID NO: 6
Rat *NPC1L1* expressed sequence tag 604034587F1 (partial sequence)	SEQ ID NO: 7
EST 603662080F1 with downstream sequences added	SEQ ID NO: 8
EST 603662080F1 with upstream and downstream sequences added	SEQ ID NO: 9
Back-translated polynucleotide sequence of rat NPC1L1	SEQ ID NO: 10
Mouse NPC1L1 polynucleotide	SEQ ID NO: 11
Mouse NPC1L1 polypeptide	SEQ ID NO: 12
Back-translated polynucleotide sequence of mouse NPC1L1	SEQ ID NO: 13
Back-translated polynucleotide sequence of human NPC1L1	SEQ ID NO: 51

5

A human NPC1L1 is also disclosed under Genbank Accession Number AF192522. As discussed below, the nucleotide sequence of the rat *NPC1L1* set forth in SEQ ID NO: 1 was obtained from an expressed sequence tag (EST) from a rat jejunum enterocyte cDNA library. SEQ ID NOs: 5-7 include partial nucleotide

sequences of three independent cDNA clones. The downstream sequence of the SEQ ID NO: 5 EST (603662080F1) were determined; the sequencing data from these experiments are set forth in SEQ ID NO: 8. The upstream sequences were also determined; these data are set forth in SEQ ID NO: 9.

5   SEQ ID NOs: 43 and 44 are the nucleotide and amino acid sequence, respectively, of human NPC1L1 which is disclosed under Genbank Accession No.: AF192522 (see Davies, *et al.*, (2000) Genomics 65(2): 137-45).

SEQ ID NO: 45 is the nucleotide sequence of a mouse *NPC1L1* which is disclosed under Genbank Accession No. AK078947.

10   NPC1L1 mediates intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. Inhibition of NPC1L1 in a patient is a useful method for reducing intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol in the patient. Reducing the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol

15   in a patient is a useful way in which to treat or prevent the occurrence of atherosclerosis, particularly diet-induced atherosclerosis.

As used herein, the term "sterol" includes, but is not limited to, cholesterol and phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol).

20   As used herein, the term "5α-stanol" includes, but is not limited to, cholestanol, 5α-campestanol and 5α-sitostanol.

Without being limited by the present hypothesis, the examples present a better understanding of the putative molecular interaction between NPC1L1 and cholesterol. In this regard, one of the more interesting features of NPC1L1 is that it

25   contains the sterol-sensing domain (SSD) originally observed in SCAP (SREBP cleavage-activating protein). SCAP controls activation of sterol regulatory element binding proteins (SREBP), a transcription factor which controls more than 35 genes related to lipid and cholesterol homeostasis (Brown, M.S. & Goldstein, J.L. A proteolytic pathway that controls the cholesterol content of membranes, cells, and

30   blood. *Proc. Natl. Acad. Sci. U.S.A.* 96, 11041-11048 (1999)). The SSD, consisting of ~180 amino acids in a packet of 5 putative membrane-spanning helices, also serves a regulatory function in two key enzymes on the cholesterol biosynthesis pathway and is present in the receptor Patched. Recently, high affinity binding of cholesterol to the

SSD on SCAP has been demonstrated (Radhakrishnan, A., Sun, L., Kwon, H.J., Brown, M.S. & Goldstein, J.L., "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)), suggesting that cholesterol may similarly bind to the SSD of

5   NPC1L1, and raising the possibility that ezetimibe may compete with cholesterol for binding at this site.

Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques

10   within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait, ed.

15   1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

20   The back-translated sequences of SEQ ID NO: 10 and of SEQ ID NO: 13 uses the single-letter code shown in Table 1 of Annex C, Appendix 2 of the PCT Administrative Instruction in the Manual of Patent Examination Procedure.

A "polynucleotide", "nucleic acid " or "nucleic acid molecule" may refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine,

25   uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide

30   sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

The term "gene" means a DNA sequence that codes for or corresponds

5   to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

10   The present invention includes nucleic acid fragments of any of SEQ ID NOs: 1, 5-11 or 13. A nucleic acid "fragment" includes at least about 30 (e.g., 31, 32, 33, 34), preferably at least about 35 (e.g, 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34), more preferably at least about 45 (e.g., 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44), and most preferably at least about 126 or more contiguous nucleotides (e.g., 130, 131,

15   132, 133, 134, 135, 136, 137, 138, 139, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 1000 or 1200) from any of SEQ ID NOs: 1, 5-11 or 13.

The present invention also includes nucleic acid fragments consisting of at least about 7 (e.g., 9, 12, 17, 19), preferably at least about 20 (e.g., 30, 40, 50, 60), more preferably about 70 (e.g., 80, 90, 95), yet more preferably at least about 100

20   (e.g., 105, 110, 114) and even more preferably at least about 115 (e.g., 117, 119, 120, 122, 124, 125, 126) contiguous nucleotides from any of SEQ ID NOs: 1, 5-11 or 13.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (e.g., 30, 40, 50, 60, 70, 80, or 90), that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an

25   mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, e.g., by incorporation of ^{32}P-nucleotides, ^{3}H-nucleotides, ^{14}C-nucleotides, ^{35}S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another

30   embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" may refer to a series of two or more amino acids in a protein, peptide or polypeptide.

"Protein", "peptide" or "polypeptide" includes a contiguous string of
5   two or more amino acids. Preferred peptides of the invention include those set forth in any of SEQ ID NOs: 2 or 12 as well as variants and fragments thereof. Such fragments preferably comprise at least about 10 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18 or 19), more preferably at least about 20 (*e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40), and yet more preferably at least about 42 (*e.g.*, 43, 44, 45, 46, 47, 48, 49, 50, 60,
10   70, 80, 90, 100, 110, 120 or 130) or more contiguous amino acid residues from any of SEQ ID NOs: 2 or 12.

The present invention also includes polypeptides, preferably antigenic polypeptides, consisting of at least about 7 (*e.g.*, 9, 10, 13, 15, 17, 19), preferably at least about 20 (*e.g.*, 22, 24, 26, 28), yet more preferably at least about 30 (*e.g.*, 32, 34,
15   36, 38) and even more preferably at least about 40 (*e.g.*, 41, 42) contiguous amino acids from any of SEQ ID NOs: 2 or 12.

The polypeptides of the invention can be produced by proteolytic cleavage of an intact peptide, by chemical synthesis or by the application of recombinant DNA technology and are not limited to polypeptides delineated by
20   proteolytic cleavage sites. The polypeptides, either alone or cross-linked or conjugated to a carrier molecule to render them more immunogenic, are useful as antigens to elicit the production of antibodies and fragments thereof. The antibodies can be used, *e.g.*, in immunoassays for immunoaffinity purification or for inhibition of NPC1L1, etc.

25   The terms "isolated polynucleotide" or "isolated polypeptide" include a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which are partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems. These components include, but are not limited to, cell membranes, cell walls, ribosomes,
30   polymerases, serum components and extraneous genomic sequences.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

"Amplification" of DNA as used herein may denote the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239: 487.

5   The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example, the expression or replication, by the cell, of a gene, a DNA or RNA sequence or a protein. Preferred host cells include HEK-293 cells, chinese hamster ovary (CHO) cells, murine macrophage J774 cells or

10   any other macrophage cell line and human intestinal epithelial Caco2 cells.

The nucleotide sequence of a nucleic acid may be determined by any method known in the art (*e.g.*, chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (1977) (Proc. Natl. Acad. Sci. USA 74: 560), in which DNA is randomly cleaved

15   using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of Sanger (Sanger, *et al.*, (1977) Proc. Natl. Acad. Sci. USA 74: 5463).

The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences,

20   including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other

25   promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site

30   (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the

invention. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, *et al.*, (1981) Nature 290: 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus

5   (Yamamoto, *et al.*, (1980) Cell 22: 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, (1981) Proc. Natl. Acad. Sci. USA 78: 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, (1982) Nature 296: 39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff, *et al.*, (1978) Proc. Natl. Acad. Sci. USA 75: 3727-3731), or the *tac* promoter

10   (DeBoer, *et al.*, (1983) Proc. Natl. Acad. Sci. USA 80: 21-25); see also "Useful proteins from recombinant bacteria" in Scientific American (1980) 242: 74-94; and promoter elements from yeast or other fungi such as the *Gal 4* promoter, the *ADC* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

15   A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded

20   by the coding sequence.

               The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. A DNA sequence is expressed in or by a cell to

25   form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

               The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant"

30   or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species.

The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

Vectors that can be used in this invention include plasmids, viruses,

5   bacteriophage, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of the host. Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, *e.g.*, Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements,

10   Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, MA.

The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems

15   include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

Expression of nucleic acids encoding the NPC1L1 polypeptides of this invention can be carried out by conventional methods in either prokaryotic or eukaryotic cells. Although *E. coli* host cells are employed most frequently in

20   prokaryotic systems, many other bacteria, such as various strains of *Pseudomonas* and *Bacillus*, are known in the art and can be used as well. Suitable host cells for expressing nucleic acids encoding the NPC1L1 polypeptides include prokaryotes and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, *e.g.*, *E. coli* and *B. subtilis*. Higher eukaryotes include established tissue

25   culture cell lines from animal cells, both of non-mammalian origin, *e.g.*, insect cells, and birds, and of mammalian origin, *e.g.*, human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. A representative vector for amplifying DNA is pBR322 or many of its derivatives (*e.g.*, pUC18 or 19). Vectors that can be used to express the

30   NPC1L1 polypeptides include, but are not limited to, those containing the *lac* promoter (pUC-series); *trp* promoter (pBR322-*trp*); *Ipp* promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as *ptac* (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, *trp*-, lac-, and *Ipp*-derived

Promoters", in Rodriguez and Denhardt (eds.) <u>Vectors: A Survey of Molecular</u>
<u>Cloning Vectors and Their Uses</u>, 1988, Buttersworth, Boston, pp. 205-236. Many
polypeptides can be expressed, at high levels, in an *E.coli*/T7 expression system as
disclosed in U.S. Patent Nos. 4,952,496; 5,693,489 and 5,869,320 and in Davanloo,

5   P., *et al.*, (1984) Proc. Natl. Acad. Sci. USA 81: 2035-2039; Studier, F.W., *et al.*,
(1986) J. Mol. Biol. 189: 113-130; Rosenberg, A. H., *et al.*, (1987) Gene 56: 125-135;
and Dunn, J.J., *et al.*, (1988) Gene 68: 259.

Higher eukaryotic tissue culture cells may also be used for the
recombinant production of the NPC1L1 polypeptides of the invention. Although any

10   higher eukaryotic tissue culture cell line might be used, including insect baculovirus
expression systems, mammalian cells are preferred. Transformation or transfection
and propagation of such cells have become a routine procedure. Examples of useful
cell lines include HeLa cells, chinese hamster ovary (CHO) cell lines, J774 cells,
HEK-293 cells, Caco2 cells, baby rat kidney (BRK) cell lines, insect cell lines, bird

15   cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually
include an origin of replication, a promoter, a translation initiation site, RNA splice
sites (if genomic DNA is used), a polyadenylation site, and a transcription termination
site. These vectors also, usually, contain a selection gene or amplification gene.
Suitable expression vectors may be plasmids, viruses, or retroviruses carrying

20   promoters derived, *e.g.*, from such sources as adenovirus, SV40, parvoviruses,
vaccinia virus, or cytomegalovirus. Examples of expression vectors include
pCR®3.1, pCDNA1, pCD (Okayama, *et al.*, (1985) Mol. Cell Biol. 5: 1136),
pMC1neo Poly-A (Thomas, *et al.*, (1987) Cell 51: 503), pREP8, pSVSPORT and
derivatives thereof, and baculovirus vectors such as pAC373 or pAC610. One

25   embodiment of the invention includes membrane bound NPC1L1. In this
embodiment, NPC1L1 can be expressed in the cell membrane of a eukaryotic cell and
the membrane bound protein can be isolated from the cell by conventional methods
which are known in the art.

The present invention also includes fusions which include the NPC1L1

30   polypeptides and *NPC1L1* polynucleotides of the present invention and a second
polypeptide or polynucleotide moiety, which may be referred to as a "tag". The
fusions of the present invention may comprise any of the polynucleotides or
polypeptides set forth in Table 1 or any subsequence or fragment thereof (discussed

above). The fused polypeptides of the invention may be conveniently constructed, for example, by insertion of a polynucleotide of the invention or fragment thereof into an expression vector. The fusions of the invention may include tags which facilitate purification or detection. Such tags include glutathione-S-transferase (GST),

5   hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as ^{32}P, ^{35}S, ^{3}H, ^{99m}Tc, ^{123}I, ^{111}In, ^{68}Ga, ^{18}F, ^{125}I, ^{131}I, ^{113m}In, ^{76}Br, ^{67}Ga, ^{99m}Tc, ^{123}I, ^{111}In and ^{68}Ga may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well

10   known in the art.

Modifications (e.g., post-translational modifications) that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications, in large part, will be determined by the host cell's post-translational

15   modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide can be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out post-translational glycosylations which are similar to those

20   of mammalian cells. For this reason, insect cell expression systems have been developed to express, efficiently, mammalian proteins having native patterns of glycosylation. An insect cell which may be used in this invention is any cell derived from an organism of the class *Insecta*. Preferably, the insect is *Spodoptera fruigiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High 5). Examples of insect expression

25   systems that can be used with the present invention, for example to produce NPC1L1 polypeptide, include Bac-To-Bac (Invitrogen Corporation, Carlsbad, CA) or Gateway (Invitrogen Corporation, Carlsbad, CA). If desired, deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems.

30   Other modifications may also include addition of aliphatic esters or amides to the polypeptide carboxyl terminus. The present invention also includes analogs of the NPC1L1 polypeptides which contain modifications, such as incorporation of unnatural amino acid residues, or phosphorylated amino acid

residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties. For example, the NPC1L1 polypeptides of the invention may be appended with a polymer which increases the half-life of the peptide in the body of a

5   subject. Preferred polymers include polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2 kDa, 5 kDa, 10 kDa, 12 kDa, 20 kDa, 30 kDa and 40 kDa), dextran and monomethoxypolyethylene glycol (mPEG).

The peptides of the invention may also be cyclized. Specifically, the amino- and carboxy-terminal residues of an NPC1L1 polypeptide or two internal

10   residues of an NPC1L1 polypeptide of the invention can be fused to create a cyclized peptide. Methods for cyclizing peptides are conventional and very well known in the art; for example, see Gurrath, *et al.*, (1992) Eur. J. Biochem. 210: 911-921.

The present invention contemplates any superficial or slight modification to the amino acid or nucleotide sequences which correspond to the

15   polypeptides of the invention. In particular, the present invention contemplates sequence conservative variants of the nucleic acids which encode the polypeptides of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants

20   of the polypeptides of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein or enzyme have been changed without altering the overall conformation and function of the polypeptide, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar

25   properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and

30   methionine; acidic amino acids, which may be interchangeable include aspartic acid and glutamic acid and basic amino acids, which may be interchangeable include histidine, lysine and arginine.

The present invention includes polynucleotides encoding rat, human or mouse NPC1L1 and fragments thereof as well as nucleic acids which hybridize to the polynucleotides. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most

5   preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, supra). The conditions of temperature

10   and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions are 55°C, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide at 42°C; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical, moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5X or 6X

15   SSC at 42°C. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5X or 6X SSC and, optionally, at a higher temperature (*e.g.*, higher than 42°C: 57°C, 59°C, 60°C, 62°C, 63°C, 65°C or 68°C). In general, SSC is 0.15M NaCl and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary

20   sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic

25   acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, supra).

30   Also included in the present invention are polynucleotides comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%,

96%, 97%, 98%, 99%, 100%) to the reference rat *NPC1L1* nucleotide (*e.g.*, any of

SEQ ID NOs: 1 or 5-10) and amino acid sequences (*e.g.*, SEQ ID NO: 2), reference

human *NPC1L1* nucleotide (*e.g.*, SEQ ID NO: 3) and amino acid sequences (*e.g.*,

SEQ ID NO: 4) or the reference mouse *NPC1L1* nucleotide (*e.g.*, any of SEQ ID

5   NOs: 11 or 13) and amino acid sequences (*e.g.*, SEQ ID NO: 12), when the

comparison is performed by a BLAST algorithm wherein the parameters of the

algorithm are selected to give the largest match between the respective sequences over

the entire length of the respective reference sequences. Polypeptides comprising

amino acid sequences which are at least about 70% similar, preferably at least about

10   80% similar, more preferably at least about 90% similar and most preferably at least

about 95% similar (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat

NPC1L1 amino acid sequence of SEQ ID NO: 2, reference human NPC1L1 amino

acid sequence of SEQ ID NO: 4 or the reference mouse NPC1L1 amino acid sequence

of SEQ ID NO: 12, when the comparison is performed with a BLAST algorithm

15   wherein the parameters of the algorithm are selected to give the largest match

between the respective sequences over the entire length of the respective reference

sequences, are also included in the present invention.

            Sequence identity refers to exact matches between the nucleotides or

amino acids of two sequences which are being compared. Sequence similarity refers

20   to both exact matches between the amino acids of two polypeptides which are being

compared in addition to matches between nonidentical, biochemically related amino

acids. Biochemically related amino acids which share similar properties and may be

interchangeable are discussed above.

            The following references regarding the BLAST algorithm are herein

25   incorporated by reference: **BLAST ALGORITHMS**: Altschul, S.F., *et al.*, (1990) J.

Mol. Biol. 215: 403-410; Gish, W., *et al.*, (1993) Nature Genet. 3: 266-272; Madden,

T.L., *et al.*, (1996) Meth. Enzymol. 266: 131-141; Altschul, S.F., *et al.*, (1997)

Nucleic Acids Res. 25: 3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7: 649-656;

Wootton, J.C., *et al.*, (1993) Comput. Chem. 17: 149-163; Hancock, J.M., *et al.*,

30   (1994) Comput. Appl. Biosci. 10: 67-70; **ALIGNMENT SCORING SYSTEMS**:

Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins" in <u>Atlas of

Protein Sequence and Structure</u>, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-

352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices

for detecting distant relationships" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219: 555-565; States, D.J., *et al.*, (1991) Methods 3: 66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89:

5   10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36: 290-300; **ALIGNMENT STATISTICS**: Karlin, S., *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87: 2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22: 2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments" in Theoretical and Computational

10   Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Protein Purification

The proteins, polypeptides and antigenic fragments of this invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification

15   tagged NPC1L1 polypeptide as discussed above), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in "Guide to Protein Purification", Methods in Enzymology, Vol.

20   182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

Purification steps can be followed by performance of assays for receptor binding activity as described below. Particularly where an NPC1L1 polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of proteolytic enzymes in the assay system, such as

25   phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

Antibody Molecules

Antigenic (including immunogenic) fragments of the NPC1L1 polypeptides of the invention are within the scope of the present invention (*e.g.*, 42 or

30   more contiguous amino acids from SEQ ID NO: 2, 4 or 12). The antigenic peptides may be useful, *inter alia*, for preparing isolated antibody molecules which recognize NPC1L1. Isolated anti-NPC1L1 antibody molecules are useful NPC1L1 ligands.

An antigen is any molecule that can bind specifically to an antibody. Some antigens cannot, by themselves, elicit antibody production. Those that can induce antibody production are immunogens.

Preferably, isolated anti-NPC1L1 antibodies recognize an antigenic

5   peptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42 (e.g., an antigen derived from rat NPC1L1). More preferably, the antibody is A0715, A0716, A0717, A0718, A0867, A0868, A1801 or A1802.

The term "antibody molecule " includes, but is not limited to, antibodies and fragments (preferably antigen-binding fragments) thereof. The term

10   includes monoclonal antibodies, polyclonal antibodies, bispecific antibodies, Fab antibody fragments, F(ab)$_2$ antibody fragments, Fv antibody fragments (e.g., V$_H$ or V$_L$), single chain Fv antibody fragments and dsFv antibody fragments. Furthermore, the antibody molecules of the invention may be fully human antibodies, mouse antibodies, rat antibodies, rabbit antibodies, goat antibodies, chicken antibodies,

15   humanized antibodies or chimeric antibodies.

Although it is not always necessary, when NPC1L1 polypeptides are used as antigens to elicit antibody production in an immunologically competent host, smaller antigenic fragments are, preferably, first rendered more immunogenic by cross-linking or concatenation, or by coupling to an immunogenic carrier molecule

20   (i.e., a macromolecule having the property of independently eliciting an immunological response in a host animal, such as diptheria toxin or tetanus). Cross-linking or conjugation to a carrier molecule may be required because small polypeptide fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, i.e.,

25   they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders them more immunogenic through what is commonly known as the "carrier effect".

Carrier molecules include, e.g., proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides,

30   etc. Protein carrier molecules are especially preferred, including, but not limited to, keyhole limpet hemocyanin and mammalian serum proteins such as human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in

the art. Preferably, the protein carrier will be foreign to the host animal in which antibodies against the fragments are to be elicited.

Covalent coupling to the carrier molecule can be achieved using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the fragments of the invention can be coupled, *e.g.*, using water-soluble carbodiimides such as dicyclohexylcarbodiimide or glutaraldehyde.

Coupling agents, such as these, can also be used to cross-link the fragments to themselves without the use of a separate carrier molecule. Such cross-linking into aggregates can also increase immunogenicity. Immunogenicity can also be increased by the use of known adjuvants, alone or in combination with coupling or aggregation.

Adjuvants for the vaccination of animals include, but are not limited to, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

Information concerning adjuvants and various aspects of immunoassays are disclosed, *e.g.*, in the series by P. Tijssen, <u>Practice and Theory of Enzyme Immunoassays</u>, 3rd Edition, 1987, Elsevier, New York. Other useful references covering methods for preparing polyclonal antisera include <u>Microbiology</u>, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, <u>Specificity of Serological Reactions</u>, 1962, Dover Publications, New York, and Williams, *et al.*, <u>Methods in Immunology and Immunochemistry</u>, Vol. 1, 1967, Academic Press, New York.

The anti-NPC1L1 antibody molecules of the invention preferably recognize human, mouse or rat NPC1L1; however, the present invention includes antibody molecules which recognize NPC1L1 from any species, preferably mammals

(e.g., cat, sheep or horse). The present invention also includes complexes comprising an NPC1L1 polypeptide of the invention and an anti-NPC1L1 antibody molecule. Such complexes can be made by simply contacting the antibody molecule with its cognate polypeptide.

5   Various methods may be used to make the antibody molecules of the invention. Human antibodies can be made, for example, by methods which are similar to those disclosed in U.S. Patent Nos. 5,625,126; 5,877,397; 6,255,458; 6,023,010 and 5,874,299.

Hybridoma cells which produce the monoclonal anti-NPC1L1
10   antibodies may be produced by methods which are commonly known in the art. These methods include, but are not limited to, the hybridoma technique originally developed by Kohler, et al., (1975) (Nature 256: 495-497), as well as the trioma technique (Hering, et al., (1988) Biomed. Biochim. Acta. 47: 211-216 and Hagiwara, et al., (1993) Hum. Antibod. Hybridomas 4: 15), the human B-cell hybridoma
15   technique (Kozbor, et al., (1983) Immunology Today 4: 72 and Cote, et al., (1983) Proc. Natl. Acad. Sci. U.S.A 80: 2026-2030), and the EBV-hybridoma technique (Cole, et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). ELISA may be used to determine if hybridoma cells are expressing anti-NPC1L1 antibodies.

20   The anti-NPC1L1 antibody molecules of the present invention may also be produced recombinantly (e.g., in an E.coli/T7 expression system as discussed above). In this embodiment, nucleic acids encoding the antibody molecules of the invention (e.g., V_H or V_L) may be inserted into a pet-based plasmid and expressed in the E.coli/T7 system. There are several methods by which to produce recombinant
25   antibodies which are known in the art. An example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567. See also Skerra, A., et al., (1988) Science 240: 1038-1041; Better, M., et al., (1988) Science 240: 1041-1043 and Bird, R.E., et al., (1988) Science 242: 423-426.

The term "monoclonal antibody," includes an antibody obtained from a
30   population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible, naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Monoclonal antibodies are

advantageous in that they may be synthesized by a hybridoma culture, essentially uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the

5   antibody by any particular method. The monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method as described by Kohler, *et al.*, (1975) Nature 256: 495.

The term "polyclonal antibody" includes an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In

10 general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes which produced non-identical antibodies. Typically, polyclonal antibodies are obtained directly from an immunized animal (*e.g.*, a rabbit).

A "bispecific antibody" comprises two different antigen binding regions which bind to distinct antigens. Bispecific antibodies, as well as methods of

15 making and using the antibodies, are conventional and very well known in the art.

Anti-idiotypic antibodies or anti-idiotypes are antibodies directed against the antigen-combining region or variable region (called the idiotype) of another antibody molecule. As disclosed by Jerne (Jerne, N. K., (1974) Ann. Immunol. (Paris) 125c: 373 and Jerne, N. K., *et al.*, (1982) EMBO 1: 234),

20 immunization with an antibody molecule expressing a paratope (antigen-combining site) for a given antigen (*e.g.*, NPC1L1) will produce a group of anti-antibodies, some of which share, with the antigen, a complementary structure to the paratope. Immunization with a subpopulation of the anti-idiotypic antibodies will, in turn, produce a subpopulation of antibodies or immune cell subsets that are reactive to the

25 initial antigen.

The term "fully human antibody" refers to an antibody which comprises human immunoglobulin sequences only. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only.

"Human/mouse chimeric antibody" refers to an antibody which

30 comprises a mouse variable region (V_H and V_L) fused to a human constant region.

"Humanized" anti-NPC1L1 antibodies are also within the scope of the present invention. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, which contain minimal sequence derived from non-

human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region of the recipient are replaced by residues from a complementary determining region of a nonhuman species (donor antibody), such as mouse, rat or

5   rabbit, having a desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are also replaced by corresponding non-human residues.

"Single-chain Fv" or "sFv" antibody fragments include the V_H and/or V_L domains of an antibody, wherein these domains are present in a single polypeptide

10   chain. Generally, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. Techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786; 5,132,405 and 4,946,778) can be adapted to produce anti-NPC1L1 specific, single chain antibodies. For a review of sFv see Pluckthun in

15   The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds., Springer-Verlag, N.Y., pp. 269-315 (1994).

"Disulfide stabilized Fv fragments" and "dsFv" include molecules having a variable heavy chain (V_H) and/or a variable light chain (V_L) which are linked by a disulfide bridge.

20   Antibody fragments within the scope of the present invention also include $F(ab)_2$ fragments which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of $F(ab)_2$ with dithiothreitol or mercaptoethylamine.

An FV fragment is a V_L or V_H region.

25   Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2.

30   The anti-NPC1L1 antibody molecules of the invention may also be conjugated to a chemical moiety. The chemical moiety may be, inter alia, a polymer, a radionuclide or a cytotoxic factor. Preferably, the chemical moiety is a polymer which increases the half-life of the antibody molecule in the body of a subject.

Suitable polymers include, but are by no means limited to, polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2kDa, 5kDa, 10kDa, 12kDa, 20kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Methods for producing PEGylated anti-IL8 antibodies which are described in U.S. Patent No.

5   6,133,426 can be applied to the production of PEGylated anti-NPC1L1 antibodies of the invention. Lee, *et al.*, (1999) (Bioconj. Chem. 10: 973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (Bioconj. Chem. 12: 545-553) discloses conjugating antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminpentaacetic acid (DTPA)).

10   The antibody molecules of the invention may also be conjugated with labels such as ^{99}Tc, ^{90}Y, ^{111}In, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{35}S, ^{51}Cr, ^{57}To, ^{226}Ra, ^{60}Co, ^{59}Fe, ^{57}Se, ^{152}Eu, ^{67}CU, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{234}Th, ^{40}K, ^{157}Gd, ^{55}Mn, ^{52}Tr or ^{56}Fe.

            The antibody molecules of the invention may also be conjugated with

15   fluorescent or chemilluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthaladehyde, fluorescamine, ^{152}Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridimium salt label, an oxalate ester

20   label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

            The antibody molecules may also be conjugated to a cytotoxic factor such as diptheria toxin, *Pseudomonas aeruginosa* exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and

25   compounds (*e.g.*, fatty acids), dianthin proteins, *Phytoiacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, crotin, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

            Any method known in the art for conjugating the antibody molecules of the invention to the various moieties may be employed, including those methods

30   described by Hunter, *et al.*, (1962) Nature 144: 945; David, *et al.*, (1974) Biochemistry 13: 1014; Pain, *et al.*, (1981) J. Immunol. Meth. 40: 219; and Nygren, J., (1982) Histochem. and Cytochem. 30: 407.

Methods for conjugating antibodies are conventional and very well
known in the art.

Screening Assays

The invention allows the identification of selective ligands of NPC1L1

5   (e.g., SEQ ID NO: 2, 4 or 12) that may be useful in treatment and management of a
variety of medical conditions, including elevated serum sterol (e.g., cholesterol) or
5α-stanol. Thus, NPC1L1 of this invention can be employed in screening systems to
identify ligands. These ligands may be agonists or antagonists of NPC1L1.
Essentially, these assays provide methods for identifying ligands of NPC1L1 by using

10   (1) NPC1L1, (2) an appropriate known NPC1L1 ligand, agonist or antagonist, for
example, a sterol (such as cholesterol, phytosterols, including, but not limited to,
sitosterol, campesterol, stigmasterol and avenosterol), a cholesterol oxidation product,
a 5α-stanol (including, but not limited to, cholestanol, 5α-campestanol and 5α-
sitostanol), a substituted azetidinone (e.g., ezetimibe), BODIPY-ezetimibe (Altmann,

15   et al., (2002) Biochim. Biophys. Acta 1580(1): 77-93) or 4", 6"-bis[(2-
fluorophenyl)carbamoyl]-beta-D-cellobiosyl derivative of 11-ketotigogenin as
described in DeNinno, et al., (1997) (J. Med. Chem. 40(16): 2547-54) or any
substituted azetidinone, and (3) a sample to be tested for the presence of a candidate
NPC1L1 ligand.

20   The term "specific" when used to describe binding of, for example, a
ligand of NPC1L1 in a screening assay is a term of art which refers to the extent by
which the ligand or antagonist (e.g., substituted azetidinone, ezetimibe, sterol (such as
cholesterol) or 5α-stanol) binds preferentially to NPC1L1 in comparison to other
proteins in the assay system. For example, detection of the specific binding of a

25   ligand of NPC1L1 binds specifically to NPC1L1 is made apparent when a signal
generated in the assay to indicate such binding exceeds, to any extent, a signal
generated in a negative control wherein, for example, NPC1L1 or ligand is absent.
Furthermore, "specific binding" includes binding of a ligand either directly to
NPC1L1 or indirectly, for example via another moiety, in a complex of which

30   NPC1L1 is a part. The moiety to which an NPC1L1 ligand binds can be another
protein or a post-translational modification of NPC1L1 (e.g., a lipid chain or a
carbohydrate chain).

Non-limiting examples of suitable substituted azetidinones for use in the screening assays include those disclosed in U.S. Patent Nos. RE37,721; 5,631,365; 5,767,115; 5,846,966; 5,688,990; 5,656,624; 5,624,920; 5,698,548; 5,756,470; 5,688,787; 5,306,817; 5,633,246; 5,627,176; 5,688,785; 5,744,467;

5 5,846,966; 5,728,827; 6,632,933 and U.S. Patent Application Publication No 2003/0105028-each of which is herein incorporated by reference in its entirety.

The present invention provides for a method by which to evaluate whether a sample contains an NPC1L1 ligand by determining whether the sample contains a candidate compound which competes for binding between the known

10 ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound 2, etc.) and NPC1L1. The ligand may be an agonist or antagonist. In an embodiment of the invention, the binding of the known ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound 2, etc.) to NPC1L1 is disrupted. The term "known ligand" refers to a compound which is known to bind to NPC1L1 and which can be detectably labeled for use in the

15 screening assays and methods described herein. "Known ligands" include the substituted 2-azetidinone glucuronides which can be detectably labeled for use in screening assays as described herein.

Ezetimibe can be prepared by a variety of methods well know to those skilled in the art, for example such as are disclosed in U.S. Patents Nos. 5,631,365,

20 5,767,115, 5,846,966, 6,207,822, U.S. Patent Application Publication No. 2002/0193607 and PCT Patent Application WO 93/02048, each of which is incorporated herein by reference in its entirety.

"Sample", "candidate compound" or "candidate substance" refers to a compound or composition which is evaluated in a test or assay, for example, for the

25 ability to bind to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) or a functional fragment thereof. The composition may comprise candidate compounds, such as small molecules, peptides, nucleotides, polynucleotides, subatomic particles (*e.g.*, α particles, β particles) or antibodies.

The present invention provides methods for identifying ligands of a

30 compound that binds to NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates

binding of the detectably labeled substituted 2-azetidinone to NPC1L1. The
modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding
of the candidate compound to NPC1L1 indicates that the candidate compound is a
ligand that binds to NPC1L1. It is also a good indication that the candidate compound
5   may be an inhibitor of sterol and 5α-stanol absorption in vivo.

The present invention also provides a method for identifying a ligand
of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-
azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the
binding of NPC1L1 of the detectably labeled substituted 2-azetidinone in the presence
10 and absence of a candidate compound, wherein decreased binding of the detectably
labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate
compound indicates that said candidate compound is a ligand of NPC1L1 and is an
inhibitor of sterol and 5α-stanol absorption.

The substituted 2-azetidinone is detectably labeled with ^3H, ^{35}S, ^{125}I, or
15 a fluorescently labeled substituted 2-azetidinone. Preferably, the substituted
2-azetidinone is labeled with ^{35}S or ^{125}I, and particularly ^{35}S.

Preferably, the substituted 2-azetidinone is substituted 2-azetidinone-
glucuronide. Compounds that are substituted 2-azetidinone-glucuronides are those
having the following structure (I):
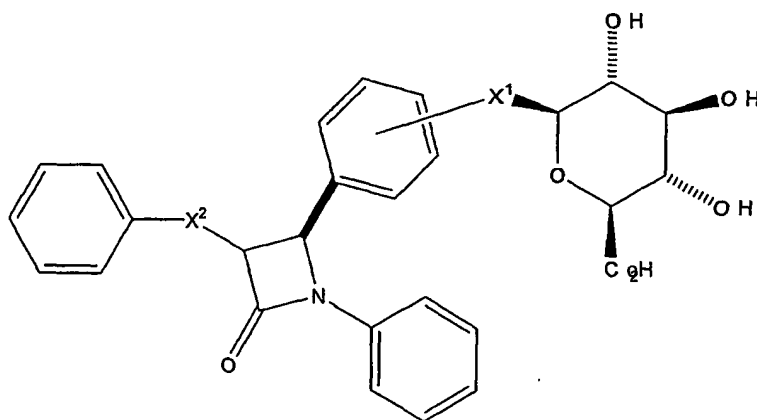
20

(I)

wherein X^1 represents a group that links the glucuronide to the
4-phenyl ring, for example but not limited to –O- or –C_{1-3} alkyl-, X^2 represents an
optionally substituted –alkanediyl-, and wherein any of the phenyl groups may be
25 optionally substituted. Examples of the phenyl-X^2-moiety in structure (I) include

those represented at the 4-position on the 2-azetidinone structure shown below in
structure (II). Additional examples of substituted 2-azetidinone-glucuronides include
but are not limited to those described in U.S. Patent No. 5,756,470, WO02/066464
and US 2002/0137689. Additional examples of substituted 2-azetidinone-glucuronide
5 compounds include those having the structure (II) and pharmaceutically acceptable
salts and esters thereof as follows:
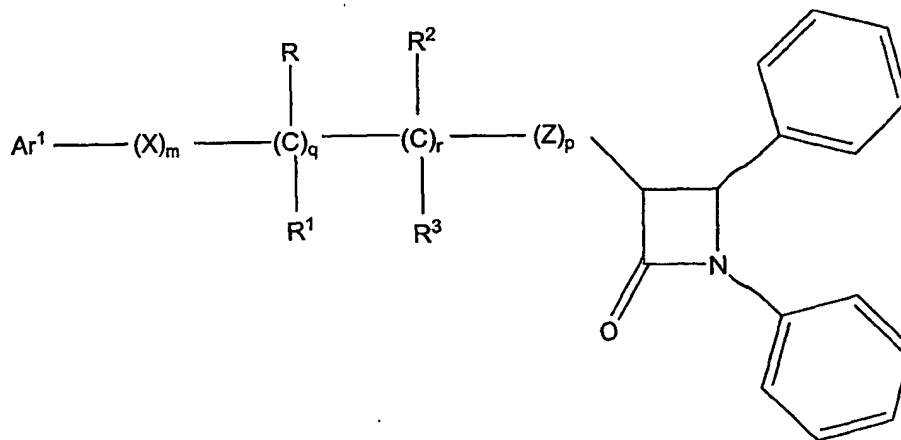
10

(II)

wherein:

Ar^1 is selected from the group consisting of aryl and R^4-substituted aryl;

X, Y and Z are independently selected from the group consisting of -CH$_2$-, -CH(C$_1$-
6alkyl)- and -C(C$_1$-6alkyl)$_2$-;

R is selected from the group consisting of -OR^6, -O(CO)R^6, -O(CO)OR^9,
-O(CO)NR^{6}R^7, a sugar residue, a disugar residue, a trisugar residue and a
tetrasugar residue;

R^1 is selected from the group consisting of -H, -C$_1$-6alkyl and aryl, or R and R^1
together are oxo;

R^2 is selected from the group consisting of -OR^6, -O(CO)R^6, -O(CO)OR^9 and
-O(CO)NR^{6}R^7;

R^3 is selected from the group consisting of -H, -C$_1$-6alkyl and aryl or R^2 and R^3
together are oxo;

q, r and t are each independently selected from 0 and 1;

m, n and p are each independently selected from 0, 1, 2, 3 and 4;

R^4 is 1-5 substituents independently selected at each occurrence from the group

consisting of:

$-OR^5$, $-O(CO)R^5$, $-O(CO)OR^8$, $-O-C_{1-5}alkyl-OR^5$, $-O(CO)NR^5R^6$, $-NR^5R^6$, $-NR^5(CO)R^6$, $-NR^5(CO)OR^8$, $-NR^5(CO)NR^6R^7$, $-NR^5SO_2R^8$, $-COOR^5$, $-CONR^5R^6$, $-COR^5$,

$-SO_2NR^5R^6$, $-S(O)_tR^8$, $-O-C_{1-10}alkyl-COOR^5$, $-O-C_{1-10}alkyl-CONR^5R^6$ and

fluoro;

R^5, R^6 and R^7 are independently selected at each occurrence from the group

consisting of -H, $C_{1-6}alkyl$, aryl and aryl-substituted $C_{1-6}alkyl$;

R^8 is independently selected from the group consisting of $C_{1-6}alkyl$, aryl and aryl-

substituted $C_{1-6}alkyl$;

R^9 is selected from the group consisting of $-C{\equiv}C-CH_2-NR^{10}R^{11}$, $-C{\equiv}C-C(O)R^{13}$,

and $-(CH_2)_3-NR^{10}R^{14}$;

R^{10} is independently selected at each occurrence from $-H$ and $-C_{1-3}alkyl$;

R^{11} is selected from the group consisting of $-H$, $-C_{1-3}alkyl$, $-C(O)-C_{1-3}alkyl$, $-C(O)-NR^{10}R^{10}$, $-SO_2-C_{1-3}alkyl$, and $-SO_2$-phenyl; and

R^{12} is selected from
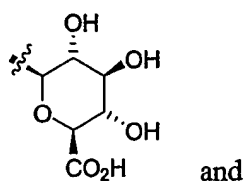
and
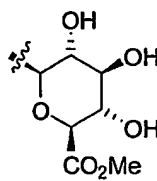
(referred to herein as "glucuronide")

(referred to herein as "methyl ester

glucuronide");

R^{13} is selected from the group consisting of $-OH$ and $-NR^{10}R^{11}$; and

R^{14} is selected from the group consisting of $-C(O)-C_{1-3}alkyl$, $-C(O)-NR^{10}R^{10}$,

$-SO_2-C_{1-3}alkyl$ and $-SO_2$-phenyl.

In one embodiment of Formula II are compounds wherein q, r and t are

each independently selected from 0 and 1; and m, n and p are each independently

selected from 0, 1, 2, 3 and 4; provided that at least one of q and r is 1, and the sum of

m, n, p, q are r is 1, 2, 3, 4, 5 or 6; and provided that when p is 0 and r is 1, the sum of

m, q and n is 1, 2, 3, 4, or 5. In a second embodiment of Formula II are compounds of

Formula IIa,

IIa
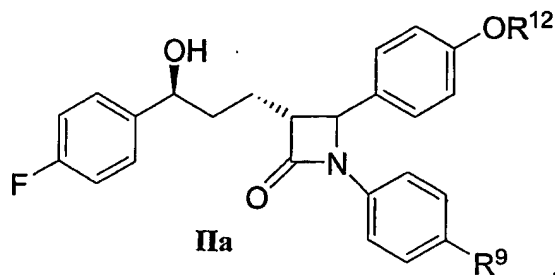
5   In a class of each of these embodiments are compounds wherein R^9 is $-C\equiv C-CH_2-$

$NR^{10}R^{11}$. In another class of each of these embodiments are compounds wherein R^9

contains an $-SO_2-$ group, i.e., wherein R^9 is selected from the group consisting of

$-C\equiv C-CH_2-NR^{10}R^{11}$, $-C\equiv C-C(O)\ NR^{10}R^{11}$, $-(CH_2)_3-NR^{10}-SO_2-C_{1-3}$alkyl and

$-(CH_2)_3-NR^{10}-SO_2-$phenyl, and R^{11} is selected from $-SO_2-C_{1-3}$alkyl, and $-SO_2-$

10   phenyl.

The term "alkyl" is intended to include both branched- and straight-

chain saturated aliphatic univalent hydrocarbon groups having the specified number

of carbon atoms. Examples of alkyl groups include, but are not limited to, methyl

(Me), ethyl (Et), n-propyl (Pr), n-butyl (Bu), n-pentyl, n-hexyl, and the isomers

15   thereof such as isopropyl (i-Pr), isobutyl (i-Bu), secbutyl (s-Bu), tertbutyl (t-Bu),

isopentyl, isohexyl and the like. If there is no specified prefix (such as "n-" for

normal, "s-" for sec, "t-" for tert, "i-" for iso) with a named alkyl group, then it is

intended that the named alkyl group is an n-alkyl group (i.e., "propyl" is "n-propyl").

The term "aryl" is intended to include phenyl (Ph), naphthyl, indenyl,

20   tetrahydronaphthyl or indanyl. Phenyl is preferred.

Suitable protecting groups (designated as "PG" herein) for the

hydroxyl groups of R^{12} when R^{12} is a glucuronide or methyl ester glucuronide

include but are not limited to those that are known to be useful as carbohydrate

protecting groups, such as for example benzyl, acetyl, benzoyl, *tert*-

25   butyldiphenylsilyl, trimethylsilyl, *para*-methoxybenzyl, benzylidine, and methoxy

methyl. Conditions required to selectively add and remove such protecting groups are

found in standard textbooks such as Greene, T, and Wuts, P. G. M., *Protective Groups in Organic Synthesis*, John Wiley & Sons, Inc., New York, NY, 1999.

Compounds of Formula II may contain one or more asymmetric centers and can thus occur as racemates and racemic mixtures, single enantiomers,

5   enantiomeric mixtures, diastereomeric mixtures and individual diastereomers, and all such isomeric forms are within the scope of Formula II.

Radioactive isotopes of the compounds of Formula II are particularly useful in such assays, for example compounds of Formula II wherein sulfur is replaced with "hot" -^{35}S-, and particularly wherein the radioactive sulfur isotope is

10   incorporated within the R^9 moiety. The use of all such radioactive isotopes of the compounds of Formula II in an assay for identifying NPC1L1 ligands is included within the scope of this invention.

The term "pharmaceutically acceptable salts" means non-toxic salts of the compounds of Formula II which are generally prepared by reacting the free acid

15   with a suitable organic or inorganic base, particularly those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc and tetramethylammonium, as well as those salts formed from amines such as ammonia, ethylenediamine, N-methylglucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine,

20   N-benzylphenethylamine, 1-p-chlorobenzyl-2-pyrrolidine-1'-yl-methylbenzimidazole, diethylamine, piperazine, morpholine, 2,4,4-trimethyl-2-pentamine and tris(hydroxymethyl)aminomethane.

When the compounds of Formula II are basic, salts may be prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic

25   acids. Such acids include acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic, lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluenesulfonic acid, and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric,

30   sulfuric, and tartaric acids.

Examples of pharmaceutically acceptable esters include, but are not limited to, -C$_{1-4}$ alkyl and -C$_{1-4}$ alkyl substituted with phenyl, dimethylamino and acetylamino. "C$_{1-4}$ alkyl" herein includes straight or branched aliphatic chains

containing from 1 to 4 carbon atoms, for example methyl, ethyl, n-propyl, n-butyl, iso-propyl, sec-butyl and tert-butyl.

The compounds of structural Formula II can be prepared according to the procedures of the following Scheme using appropriate materials, and are further

5 exemplified by specific examples which follow. A variety of chromatographic techniques may be employed in the preparation of the compounds of Formula II. These techniques include, but are not limited to: High Performance Liquid Chromatography (including normal- reversed- and chiral-phase); Super Critical Fluid Chromatography; preparative Thin Layer Chromatography; flash chromatography

10 with silica gel or reversed-phase silica gel; ion-exchange chromatography; and radial chromatography. All temperatures are degrees Celsius unless otherwise noted.

Some abbreviations used herein include:

Ac	Acyl ($CH_3C(O)$-)
Bn	benzyl
calc.	Calculated
Celite	Celite^{TM} diatomaceous earth
Dess-Martin Periodinane	1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benzodoxol-3-($1H$)-one
DMF	N,N-dimethylformamide
equiv.	Equivalent(s)
ES-MS	Electron Spray Ion-Mass Spectroscopy
EtOAc	Ethyl acetate
h	Hour(s)
HPLC	High performance liquid chromatography
min	Minute(s)
m.p.	Melting point
MS	Mass spectrum
r.t. (or rt)	Room temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tlc	Thin layer chromatography

The general Scheme below illustrates a method for the syntheses of

15 compounds of structural formula II-4. All substituents are as defined in Formula II unless indicated otherwise. In this method, II-1 is treated with a terminal alkyne of

type II-2 in the presence of a suitable palladium catalyst such as tetrakistriphenylphosphine palladium(0) or [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) or the like, and copper(I) iodide. The reaction is usually performed in an inert organic solvent such as DMF,

5   between room temperature and 100 °C, for a period of 6-48 h, and the product is an internal alkyne of structural formula II-3. Alkyne II-2 may contain a radioactive atom such as 35S to provide the corresponding radiolabeled adduct upon reaction with II-1. Conversion of II-3 to II-4 can be achieved using a variety of hydrolytic methods known to those skilled in the art of organic synthesis. For example, a particularly

10   mild hydrolysis protocol involves the treatment of II-3 with a tertiary amine base such as triethylamine, or diisopropylethylamine or the like, in a mixed solvent system comprising methanol and water. The product of the reaction is a compound of structural formula II-4. By utilizing the procedures described herein, one of ordinary skill in the art can readily prepare additional compounds of Formual II.

15   SCHEME
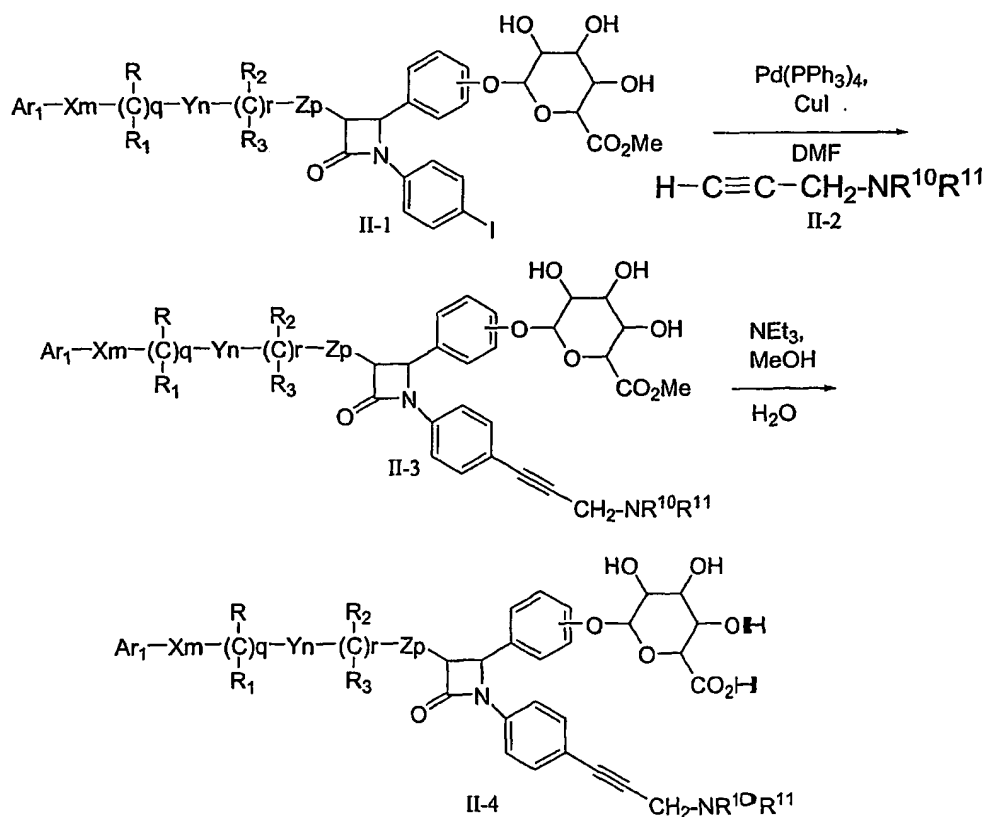
Two additional types of screening systems that can be used include a labeled-ligand binding assay (*e.g.*, direct binding assay or scintillation proximity assay (SPA)) and a "sterol (*e.g.*, cholesterol) or 5α-stanol uptake" assay. A labeled ligand, for use in the binding assay, can be obtained by labeling a sterol (*e.g.*,

5   cholesterol) or a 5α-stanol or a known NPC1L1 agonist or antagonist with a measurable group (*e.g.*, ^{35}S, ^{125}I or ^{3}H). Various labeled forms of sterols (*e.g.*, cholesterol) or 5α-stanols are available commercially or can be generated using standard techniques (*e.g.*, Cholesterol- [1,2-^{3}H(N)], Cholesterol-[1,2,6,7-^{3}H(N)] or Cholesterol-[7-^{3}H(N)]; American Radiolabeled Chemicals, Inc; St. Louis, MO). In a

10   preferred embodiment, ezetimibe is fluorescently labeled with a BODIPY group (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) or labeled with a detectable group such as ^{35}S, ^{125}I or ^{3}H, preferably ^{35}S.

Direct Binding Assay. Typically, a given amount of NPC1L1 of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) or a complex including NPC1L1 is contacted

15   with increasing amounts of labeled ligand or known antagonist or agonist (discussed above) and the amount of the bound, labeled ligand or known antagonist or agonist is measured after removing unbound, labeled ligand or known antagonist or agonist by washing. As the amount of the labeled ligand or known agonist or antagonist is increased, a point is eventually reached at which all receptor binding sites are

20   occupied or saturated. Specific receptor binding of the labeled ligand or known agonist or antagonist is abolished by a large excess of unlabeled ligand or known agonist or antagonist.

Preferably, an assay system is used in which non-specific binding of the labeled ligand or known antagonist or agonist to the receptor is minimal. Non-

25   specific binding is typically less than 50%, preferably less than 15%, more preferably less than 10%, and most preferably 5% or less, of the total binding of the labeled ligand or known antagonist or agonist.

In the basic binding assay, the method for identifying an NPC1L1 ligand, agonist or antagonist includes:

30   (a)   contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1, in the presence of a known amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist (*e.g.*,

labeled ezetimibe) with a sample to be tested for the presence of an NPC1L1 ligand, agonist or antagonist; and

(b)   measuring the amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist directly or indirectly bound to NPC1L1.

5   An NPC1L1 ligand in the sample is identified by measuring substantially reduced direct or indirect binding of the labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist to NPC1L1, compared to what would be measured in the absence of such a ligand. For example, reduced direct or indirect binding between [^{3}H]-cholesterol and NPC1L1 in the presence of a sample might

10   suggest that the sample contains a substance which is competing against [^{3}H]-cholesterol for NPC1L1 binding.

This assay can include a control experiment lacking any NPC1L1-dependent ligand (*e.g.*, sterol such as cholesterol or 5α-stanol) binding. In this assay, for example, a whole cell or cell membrane lacking any functional NPC1L1, for

15   example, a cell or membrane isolated or derived from a transgenic mutant *npc1l1⁻* mouse of the invention, is assayed for ligand binding. When screening a sample for the presence of an NPC1L1 antagonist, it is useful to compare the level of binding observed in the presence of a sample being tested with that of a control experiment, as described herein, which completely lacks NPC1L1-dependent binding. Ideally,

20   though by no means necessarily, the level of binding seen in the presence of a sample containing an antagonist will be similar to that of the control experiment.

Alternatively, a sample can be tested directly for binding to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12). A basic assay of this type may include the following steps:

25   (a)   contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1 with a labeled candidate compound (*e.g.*, [^{3}H]-ezetimibe); and

(b)   detecting direct or indirect binding between the labeled candidate compound and NPC1L1.

30   Again, these experiment can be performed along with a control experiment wherein NPC1L1-dependent binding is completely lacking. For example, the assay can be performed using a whole cell or cell membrane lacking any

functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant *npc1l1⁻* mouse as described herein).

A candidate compound which is found to bind to NPC1L1 may function as ligand, agonist or antagonist of NPC1L1 (*e.g.*, by inhibition of sterol
5   (*e.g.*, cholesterol) or 5α-stanol uptake).

In an embodiment of the invention, the bound candidate compound is quantified after filtration using glass fiber filters. In one aspect of this embodiment, the bound candidate compound is detected after single-tube vacuum filtration of GF/C glass fiber filters, obtained from Whatman. The filters may be pretreated by soaking
10   with 0.5% polyethylenimine to reduce nonspecific binding. Filtration is accomplished by adding ice cold buffer to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with additional buffer. The buffer may be a Tris buffer or MES buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70). The filters can be counted using scintillation fluid, e.g., Packard DM
15   liquid or Packard Ultima Gold MV.

Alternatively, vacuum filtration of the sample on a Milliore 96-well plate (Whatman GF/C) can also be used to achieve adequate precision in a manner well-known to those skilled in the art.

SPA Assay. NPC1L1 ligands may also be measured using scintillation
20   proximity assays (SPA). SPA assays are conventional and very well known in the art; see, for example, U.S. Patent No. 4,568,649. In SPA, the target of interest is immobilized to a small microsphere approximately 5 microns in diameter. The microsphere, typically, includes a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules, which allow generic
25   links for assay design. When a radioisotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from electrons emitted by the isotope will take place resulting in the emission of light. While the radioisotope remains in free solution, it is too distant from the scintillant and the electron will dissipate the energy into the
30   aqueous medium and therefore remain undetected. Scintillation may be detected with a scintillation counter. In general, ^{3}H, ^{125}I and ^{35}S labels are well suited to SPA.

For the assay of receptor-mediated binding events, the lectin wheat germ agglutinin (WGA) may be used as the SPA bead coupling molecule (Amersham

Biosciences; Piscataway, NJ). The WGA coupled bead captures glycosylated, cellular membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA-SPA bead and a signal is generated on binding of an isotopically labeled

5   ligand. Other coupling molecules which may be useful for receptor binding SPA assays include poly-L-lysine and WGA/polyethyleneimine (Amersham Biosciences; Piscataway, NJ). See, for example, Berry, J.A., *et al.*, (1991) Cardiovascular Pharmacol. 17 (Suppl.7): S143-S145; Hoffman, R., *et al.*, (1992) Anal. Biochem. 203: 70-75; Kienhus, *et al.*, (1992) J. Receptor Research 12: 389-399; Jing, S., *et al.*,

10   (1992) Neuron 9: 1067-1079.

        The scintillant contained in SPA beads may include, for example, yttrium silicate (YSi), yttrium oxide (YOx), diphenyloxazole or polyvinyltoluene (PVT) which acts as a solid solvent for diphenylanthracine (DPA).

        SPA assays may be used to analyze whether a sample contains an

15   NPC1L1 ligand. In these assays, a host cell which expresses NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface or a membrane fraction thereof is incubated with and captured by SPA beads (*e.g.*, WGA coated YOx beads or WGA coated YSi beads). The beads bearing the NPC1L1 are incubated with labeled, known ligand or agonist or antagonist (*e.g.*, ^{3}H-cholesterol, ^{3}H-ezetimibe, ^{125}I-ezetimibe or a ^{35}S-

20   ezetimibe analog). The assay mixture further includes either the sample to be tested or a blank (*e.g.*, water). After an optional incubation, scintillation is measured using a scintillation counter. An NPC1L1 ligand, agonist or antagonist may be identified in the sample by measuring substantially reduced fluorescence, compared to what would be measured in the absence of such ligand, agonist or antagonist (blank). Measuring

25   substantially reduced fluorescence may suggest that the sample contains a substance which competes for direct or indirect NPC1L1 binding with the known ligand, agonist or antagonist.

        Alternatively, a sample may be identified as an ligand of NPC1L1 by directly detecting binding in a SPA assay. In this assay, a labeled version of a

30   candidate compound to be tested may be put in contact with the host cell expressing NPC1L1 or a membrane fraction thereof which is bound to the SPA bead. Fluorescence may then be assayed to detect the presence of a complex between the labeled candidate compound and the host cell or membrane fraction expressing

NPC1L1 or a complex including NPC1L1. A candidate compound which binds directly or indirectly to NPC1L1 may possess NPC1L1 agonistic or antagonistic activity.

SPA Assays can also be performed along with a control experiment
5   lacking any NPC1L1-dependent binding. The control experiment can be performed, for example, with a cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant npc1l1- mouse as described herein). When the control experiment is performed, the level of binding observed in the presence of sample being tested for the presence of an antagonist can be compared
10   with that observed in the control experiment.

Sterol/5α-stanol Uptake Assay. Assays may also be performed to determine if a sample can agonize or antagonize NPC1L1 mediated sterol (*e.g.*, cholesterol) or 5α-stanol uptake. In these assays, a host cell expressing NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface (discussed above) can be contacted
15   with detectably labeled sterol (*e.g.*, ^{3}H-cholesterol or ^{125}I-cholesterol)) or 5α-stanol along with either a sample or a blank. After an optional incubation, the cells can be washed to remove unabsorbed sterol or 5α-stanol. Sterol or 5α-stanol uptake can be determined by detecting the presence of labeled sterol or 5α-stanol in the host cells. For example, assayed cells or lysates or fractions thereof (*e.g.*, fractions resolved by
20   thin-layer chromatography) can be contacted with a liquid scintillant and scintillation can be measured using a scintillation counter.

In these assays, an NPC1L1 antagonist in the sample may be identified by measuring substantially reduced uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an antagonist
25   and an agonist may be identified by measuring substantially increased uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an agonist.

Uptake assays can also be performed along with a control experiment lacking any NPC1L1-dependent uptake. The control experiment can be performed,
30   for example, with a cell lacking any functional NPC1L1 (*e.g.*, cell derived from a transgenic, mutant *npc1l1⁻* mouse as described herein). When the control experiment is performed, the level of uptake observed in the presence of sample being tested for

the presence of an antagonist can be compared with that observed in the control experiment.

Source of NPC1L1. In principle, a binding assay of the invention could be carried out using a soluble NPC1L1 polypeptide of the invention, *e.g.,*
5 following production and refolding by standard methods from an *E. coli* or other prokaryotic or eukaryotic expression system, and the resulting receptor-labeled ligand complex could be precipitated, *e.g.,* using an antibody against the receptor. The precipitate could then be washed and the amount of the bound, labeled ligand or antagonist or agonist could be measured.

10 Alternatively, NPC1L1 is membrane-bound. A nucleic acid encoding an NPC1L1 polypeptide of the invention (*e.g.,* SEQ ID NO: 2, 4 or 12) can be transfected into an appropriate host cell, whereby the NPC1L1 will become incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of NPC1L1 for assay. Alternatively, the whole cell
15 expressing NPC1L1 in the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or to a membrane fraction from an untransfected/untransformed host cell will be negligible.

Various membranes may be used directly as a source of NPC1L1 for
20 the above-described screening systems, e.g. direct binding, scintillation proximity assay, sterol/5α stanol uptake assay. As described in Examples 5, 6, 7, 8, 9, 17, 27, and 29, NPC1L1 is highly expressed in certain tissues, especially in brush border cells of intestinal tissues. Therefore, brush border membrane (BBM) vesicle preparations may be utilized as a source of NPC1L1. The membranes may be derived from
25 mammalian intestinal tissue from rhesus, rat, mouse or human tissue.

Membranes may be derived from brush border cells of intestinal tissues. Such membranes are conventionally prepared by collecting intestinal tissue from freshly sacrificed animals. The mucosa of the tissue is scraped, collected into buffered solutions, and homogenized. Cellular debris is removed and the membrane
30 fractions are collected by centrifugation. Conventional techniques known to one of skill in the art maybe used for the preparation of brush border membrane vesicles. *See* Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal,

K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993);
Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992).

   The membrane preparation may be in vesicular or non-vesicular form.

   Alternatively, liposomes and liposomal preparations comprising

5 NPC1L1 may also be a viable source of NPC1L1 for the screening assays of the
present claimed method.

   In vitro cultured cells expressing NPC1L1 may also be used. The host
cells may be prepared by transforming or transfecting a nucleic acid encoding an
NPC1L1 of the invention into an appropriate host cell, whereby the receptor becomes

10 incorporated into the membrane of the cell. A membrane fraction can then be isolated
from the cell and used as a source of the receptor for assay. Alternatively, the whole
cell expressing the receptor on the cell surface can be used in an assay. Preferably,
specific binding of the labeled ligand or known antagonist or agonist to an
untransfected/untransformed host cell or membrane fraction from an

15 untransfected/untransformed host cell will be negligible.

   Preferred host cells include Chinese Hamster Ovary (CHO) cells,
murine macrophage J774 cells, HEK-293 cells or any other macrophage cell line and
human intestinal epithelial Caco2 cells.

   The present invention provides for a method of identifying a ligand of

20 NPC1L1 using these membrane preparations, for example by contacting membranes
comprising NPC1L1, such as brush border membrane vesicle preparations, with
detectably labeled substituted azetidinone compounds which are known NPC1L1
ligands, agonists or antagonists, and a candidate compound and determining whether
the candidate compound can bind to NPC1L1. The binding of the candidate

25 compound to NPC1L1 may modulate binding of the detectably labeled NPC1L1
ligands, agonists or antagonists to NPC1L1. In addition, a NPC1L1 ligand may be
identified by measuring the binding of NPC1L1 with detectably labeled NPC1L1
ligands, agonists or antagonists in the presence and absence of the candidate
compound wherein decreased binding of the detectably labeled NPC1L1 ligands,

30 agonists or antagonists to NPC1L1 is an indication that the candidate compound is
ligand of NPC1L1.

NPC1L1 may also be obtained by solubilization of membrane fractions comprising NPC1L1. The membranes may be obtained as discussed above, *e.g.*, from mammalian tissue or *in vitro* cultured cells.

   Binding Affinities of NPC1L1 Ligands. The affinity and specificity of
5 the known ligand (e.g., detectably labeled substituted 2-azetidinone-glucuronide) for NPC1L1 are important to the identification of ligands that bind NPC1L1 in a screening assay. It is understood that the known ligand will be labeled for use in the screening assay. In an embodiment of the invention, the binding affinity of the known ligand for human NPC1L1 has a K_D value equivalent or lower than the K_D value of
10 ezetimibe glucuronide $\underline{1}$ for human NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for human NPC1L1 has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or lower; even more particularly it has a K_D value of about 20nM or lower; and most particularly it has a K_D value of about
15 10nM or lower. For usefulness in the assay, there is essentially no lower limit on the K_D value of the known ligand and it may, for example, go down into the pM range. As the K_D value decreases, the binding affinity of the ligand for human NPC1L1 increases, which is desirable for the screening assay.

   In another embodiment of the invention, the binding affinity of the
20 known ligand for rat NPC1L1 has a K_D value equivalent or lower than the K_D value of ezetimibe glucuronide $\underline{1}$ for rat NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for rat NPC1L1 has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or lower; even more particularly it has a K_D value of
25 about 20nM or lower; and most particularly it has a K_D value of about 10nM or lower.

   In another embodiment of this invention, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II wherein R^9 contains an $-SO_2-$ group and (b) a substituted 2-azetidinone-glucuronide
30 labeled with [125]I.

   In one aspect of this embodiment, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II

wherein R^9 contains an $-SO_2-$ group, and (b) a substituted 2-azetidinone-glucuronide labeled with ^{125}I, and has a K_D value equivalent or lower than the K_D value of ezetimibe glucuronide 1.

In another aspect of this embodiment, the known ligand for human
5   NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II wherein R^9 contains an $-SO_2-$ group, and (b) a substituted 2-azetidinone-glucuronide labeled with ^{125}I, and has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or
10   lower; even more particularly it has a K_D value of about 20nM or lower; and most particularly it has a K_D value of about 10nM or lower.

When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands from among the candidate compounds using mouse-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those
15   candidates that exhibit a binding affinity having a K_D value of about 12,000nM or lower, preferably about 1000nM or lower, more preferably about 100nM or lower, and most preferably about 10nM or lower. When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rat-derived membranes or human-derived membranes, candidate compounds identified as NPC1L1 ligands are
20   preferably those candidates that exhibit a binding affinity having a K_D value of about 1000nM or lower, preferably about 100nM or lower, and more preferably about 10nM or lower. When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rhesus monkey-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding
25   affinity having a K_D value of about 50nM or lower, and preferably about 10nM or lower.

When using ^{35}S-labeled compound 2 in screening to identify NPC1L1 ligands from among the candidate compounds using rat or human-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those
30   candidates that exhibit a binding affinity having a K_D value in the range from about 10μM to about 1nM. When using ^{125}I-labeled substituted 2-azetidinone glucuronide compounds in the assay with rat or human membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding

affinity having a K_D value in the range from about 10nM to about 10pM, and preferably from about 100pM to about 10pM.

Mouse Assay. The present invention comprises a mutant, transgenic mouse which lacks any functional NPC1L1. This mouse may serve as a convenient
5   control experiment in screening assays for identifying inhibitors of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, preferably inhibitors of NPC1L1. Preferably, a mouse assay of the present invention would comprise the following steps:

          (a)   feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing
10   substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional NPC1L1;

          The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance preferably contains labeled cholesterol, such as a radiolabeled cholesterol, for
15   example, ^{3}H or ^{14}C labeled cholesterol. The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance may also include cold, unlabeled sterol (*e.g.*, cholesterol) or 5α-stanol such as in corn oil.

          In these assays, the third *npc1l1⁻* mutant mouse serves as a (+)-control experiment which exhibits low levels of intestinal sterol (*e.g.*, cholesterol) or 5α-
20   stanol absorption and the second mouse serves as a (-)-control experiment which exhibits normal, uninhibited levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. The second mouse is not administered the sample to be tested for an NPC1L1 antagonist. The first mouse is the experiment.

          (b)   administering the sample to the first mouse comprising a
25   functional *NPC1L1* but not to the second mouse;

          (c)   measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse;

          Intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption may be measured by any method known in the art. For example, the level intestinal
30   absorption can be assayed by measuring the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol.

          (d)   comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse;

wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and in the third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the

5   second mouse.

Preferably, if the sample contains an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption inhibitor (*e.g.*, an NPC1L1 inhibitor), the level of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse will be similar to that of the third, *npc1l1* mutant mouse.

10   An alternative, (+)-control experiment which may be used in these screening assays is a mouse comprising functional NPC1L1 which is administered a known antagonist of NPC1L1, such as ezetimibe.

Pharmaceutical Compositions

NPC1L1 ligands discovered, for example, by the screening methods

15   described above may be used therapeutically (*e.g.*, in a pharmaceutical composition) to stimulate or block the activity of NPC1L1 and, thereby, to treat any medical condition caused or mediated by NPC1L1. In addition, the antibody molecules of the invention may also be used therapeutically (*e.g.*, in a pharmaceutical composition) to bind NPC1L1 and, thereby, block the ability of NPC1L1 to bind a sterol (*e.g.*,

20   cholesterol) or 5α-stanol. Blocking the binding of a sterol (*e.g.*, cholesterol) or 5α-stanol would prevent absorption of the molecule (*e.g.*, by intestinal cells such as enterocytes). Blocking absorption of sterol (*e.g.*, cholesterol) or 5α-stanol would be a useful way to lower serum sterol (*e.g.*, cholesterol) or 5α-stanol levels in a subject and, thereby, reduce the incidence of, for example, hyperlipidemia, atherosclerosis,

25   coronary heart disease, stroke or arteriosclerosis.

The term "subject" or "patient" includes any organism, preferably animals, more preferably mammals (*e.g.*, mice, rats, rabbits, dogs, horses, primates, cats) and most preferably humans.

The term "pharmaceutical composition" refers to a composition

30   including an active ingredient and a pharmaceutically acceptable carrier and/or adjuvant.

Although the compositions of this invention could be administered in simple solution, they are more typically used in combination with other materials such

as carriers, preferably pharmaceutically acceptable carriers. Useful, pharmaceutically
acceptable carriers can be any compatible, non-toxic substances suitable for
delivering the compositions of the invention to a subject. Sterile water, alcohol, fats,
waxes, and inert solids may be included in a pharmaceutically acceptable carrier.

5   Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also
be incorporated into the pharmaceutical composition.

        Preferably, the pharmaceutical compositions of the invention are in the
form of a pill or capsule. Methods for formulating pills and capsules are very well
known in the art. For example, for oral administration in the form of tablets or

10   capsules, the active drug component may be combined with any oral, non-toxic
pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose,
magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl
alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable
binders, lubricants, disintegrating agents and coloring agents may also be incorporated

15   in the mixture. Suitable binders include starch, gelatin, natural sugars, corn
sweeteners, natural and synthetic gums such as acacia, sodium alginate,
carboxymethylcellulose, polyethylene glycol and waxes. Among the lubricants there
may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium
acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose,

20   guar gum and the like. Sweetening and flavoring agents and preservatives may also
be included where appropriate.

        The pharmaceutical compositions of the invention may be
administered in conjunction with a second pharmaceutical composition or substance.
In preferred embodiments, the second composition includes a cholesterol-lowering

25   drug. When a combination therapy is used, both compositions may be formulated
into a single composition for simultaneous delivery or formulated separately into two
or more compositions (*e.g.*, a kit).

        The formulations may conveniently be presented in unit dosage form
and may be prepared by any methods well known in the art of pharmacy. See, *e.g.*,

30   Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics*, 8th Ed.,
Pergamon Press; and *Remington's Pharmaceutical Sciences, supra,* Easton, Penn.;
Avis *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*
Dekker, New York; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms:*

Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, New York.

The dosage regimen involved in a therapeutic application may be determined by a physician, considering various factors which may modify the action

5   of the therapeutic substance, *e.g.*, the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration, and other clinical factors. Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Dosages may be adjusted to account for the smaller molecular sizes and possibly decreased half-lives (clearance times) following administration.

10   An "effective amount" of a ligand of the invention may be an amount that will detectably reduce the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption or detectably reduce the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol in a subject administered the composition.

Typical protocols for the therapeutic administration of such substances

15   are well known in the art. Pharmaceutical composition of the invention may be administered, for example, by any parenteral or non-parenteral route.

Pills and capsules of the invention can be administered orally. Injectable compositions can be administered with medical devices known in the art; for example, by injection with a hypodermic needle.

20   Injectable pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

Anti-Sense

25   The present invention also encompasses anti-sense oligonucleotides capable of specifically hybridizing to mRNA encoding NPC1L1 (*e.g.*, any of SEQ ID NOs: 1, 3, 5-11 or 13) having an amino acid sequence defined by, for example, SEQ ID NO: 2 or 4 or 12 or a subsequence thereof so as to prevent translation of the mRNA. Additionally, this invention contemplates anti-sense oligonucleotides capable

30   of specifically hybridizing to the genomic DNA molecule encoding NPC1L1, for example, having an amino acid sequence defined by SEQ ID NO: 2 or 4 or 12 or a subsequence thereof.

This invention further provides pharmaceutical compositions comprising (a) an amount of an oligonucleotide effective to reduce NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5α-stanol absorption by passing through a cell membrane and binding specifically with mRNA encoding NPC1L1 in the cell so as to prevent its

5   translation and (b) a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the oligonucleotide is coupled to a substance that inactivates mRNA. In another embodiment, the substance that inactivates mRNA is a ribozyme.

Reducing the level of NPC1L1 expression by introducing anti-sense

10 NPC1L1 RNA into the cells of a patient is a useful method reducing intestinal sterol (*e.g.*, cholesterol) or 5☐-stanol absorption and serum cholesterol in the patient.

Kits

Kits of the present invention include ezetimibe, preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more

15 preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. See for example, Gilman *et al.* (eds.) (1990), The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, supra, Easton, Penn.; Avis *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications

20 Dekker, New York; Lieberman *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York. Preferably, the dosage form is a Zetia® tablet (Merck/Schering-Plough Corp.). Ezetimibe may be supplied in any convenient form. For example, tablets including ezetimibe may be supplied in bottles

25 of 30, 90 or 500.

The kits of the present invention also include information, for example in the form of a package insert, indicating that the target of ezetimibe is NPC1L1 (NPC3). The term "target of ezetimibe" indicates that ezetimibe reduces intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, either directly or indirectly, by

30 antagonizing NPC1L1. The form of the insert may take any form, such as paper or on electronic media such as a magnetically recorded medium (*e.g.*, floppy disk) or a CD-ROM.

The package insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following

5   information regarding ezetimibe (*e.g.*, Zetia®) and/or simvastatin (*e.g.*, Zocor®) may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

10   The kits of the invention may also include simvastatin (
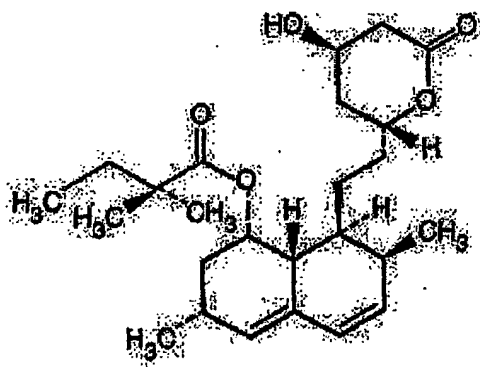
) preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a

15   suppository. Preferably, the dosage form of simvastatin is a Zocor® tablet (Merck & Co.; Whitehouse Station, NJ).

Tablets or pills comprising simvastatin may be supplied in any convenient form. For example, pills or tablets comprising 5mg simvastatin can be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 10

20   mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 20 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 40 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 80 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

Ezetimibe and simvastatin may be supplied, in the kit, as separate compositions or combined into a single composition. For example, ezetimibe and simvastatin may be supplied within a single, common pharmaceutical dosage form (*e.g.*, pill or tablet) as in separate pharmaceutical dosage forms (*e.g.*, two separate pills or tablets).

npc1l1⁻ Cells

The present invention provides any isolated mammalian cell, (*e.g.*, an isolated mouse cell, an isolated rat cell or an isolated human cell) which lacks an NPC1L1 gene which encodes or can produce a functional NPC1L1 protein. Included within this embodiment are mutant *npc1l1* genes comprising a point mutation, truncation or deletion of the genetic coding region or of any regulatory element (*e.g.*, a promoter).

For example, the cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal NPC1L1 wherein the mouse does not produce any functional NPC1L1 protein (*e.g.*, the mouse described below in Example 22). Moreover, the present invention comprises any cell, tissue, organ, fluid, nucleic acid, peptide or other biological substance derived or isolated from such a mutant mouse, particularly a mutant, transgenic mouse which does not produce any functional NPC1 L1, wherein the region of endogenous, chromosomal NPC1L1 deleted, in the mouse, corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ ID NO: 45.

The isolated cell can be isolated or derived, for example, from the duodenum, gall bladder, liver, small intestine or stomach of the mutant mouse. Further, the cell can be an enterocyte.

The *npc1l1⁻* mutant cells are useful, for example, for use in control experiments in screening assays (see *e.g.*, supra) since they lack any NPC1L1-dependent uptake or binding of sterol, 5α-stanol or ezetimibe. The level of inhibition caused by a particular sample, in a screening assay, can be compared to that of an assay performed with the mutant cell. Ideally, though by no means necessarily, in a screening assay, for example, as described herein, the same amount of binding will be observed by a non-mutant cell or cell membrane, in the presence of an antagonist, as is observed in connection with a mutant *npc1l1* cell or cell membrane alone.

EXAMPLES

The following examples are provided to more clearly describe the present invention and should not be construed to limit the scope of the invention in any way.

5   **Example 1: Cloning and Expression of Rat, Mouse and Human NPC1L1.**

Rat *NPC1L1*, mouse *NPC1L1* or human *NPC1L1* can all conveniently be amplified using polymerase chain reaction (PCR). In this approach, DNA from a rat, mouse or human cDNA library can be amplified using appropriate primers and standard PCR conditions. Design of primers and optimal amplification conditions

10   constitute standard techniques which are commonly known in the art.

An amplified *NPC1L1* gene may conveniently be expressed, again, using methods which are commonly known in the art. For example, NPC1L1 may be inserted into a pET-based plasmid vector (Stratagene; La Joola, CA), downstream of the T7 RNA polymerase promoter. The plasmid may then be transformed into a T7

15   expression system (*e.g.*, BL21DE3 E.coli cells), grown in a liquid culture and induced (*e.g.*, by adding IPTG to the bacterial culture).

Example 2: Direct Binding Assay.

Membrane preparation: Caco2 cells transfected with an expression vector containing a polynucleotide encoding NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12)

20   are harvested by incubating in 5 mM EDTA/phosphate-buffered saline followed by repeated pipeting. The cells are centrifuged 5 min at 1000 x g. The EDTA/PBS is decanted and an equal volume of ice-cold 50mM Tris-HCl, pH 7.5 is added and cells are broken up with a Polytron (PT10 tip, setting 5, 30 sec). Nuclei and unbroken cells are sedimented at 1000 x g for 10 min and then the supernatant is centrifuged at

25   50,000 x g for 10 min. The supernatant is decanted, the pellet is resuspended by Polytron, a sample is taken for protein assay (bicinchoninic acid, Pierce), and the tissue is again centrifuged at 50,000 x g. Pellets are stored frozen at –20°C.

Binding assay: For saturation binding, four concentrations of [^{3}H]-ezetimibe (15 Ci/mmol) are incubated without and with 10^{-5} M ezetimibe in triplicate

30   with 50 μg of membrane protein in a total volume of 200 μl of 50 mM Tris-HCl, pH 7.5, for 30 min at 30°C. Samples are filtered on GF/B filters and washed three times with 2 ml of cold Tris buffer. Filters are dried in a microwave oven, impregnated

with Meltilex wax scintillant, and counted at 45% efficiency. For competition

binding assays, five concentrations of a sample are incubated in triplicate with 18 nM

[^{3}H]-ezetimibe and 70 μg of membrane protein under the conditions described above.

Curves are fit to the data with Prism (GraphPad Software) nonlinear least-squares

5   curve-fitting program and K_i values are derived from IC_{50} values according to Cheng

and Prusoff (Cheng, Y. C., *et al.*, (1973) Biochem. Pharmacol. 22: 3099-3108).

Example 3A: SPA Assay.

For each well of a 96 well plate, a reaction mixture of 10 μg human,

mouse or rat NPC1L1-CHO overexpressing membranes (Biosignal) and 200 μg/well

10   YSi-WGA-SPA beads (Amersham) in 100 μl is prepared in NPC1L1 assay buffer

(25 mM HEPES, pH 7.8, 2 mM CaCl$_2$, 1mM MgCl$_2$, 125 mM NaCl, 0.1% BSA). A

0.4 nM stock of ligand- [^{125}I]-ezetimibe- is prepared in the NPC1L1 assay buffer. The

above solutions are added to a 96-well assay plate as follows: 50 μl NPC1L1 assay

buffer, 100 μl of reaction mixture, 50 μl of ligand stock (final ligand concentration is

15   0.1 nM). The assay plates are shaken for 5 minutes on a plate shaker, then incubated

for 8 hours before cpm/well are determined in Microbeta Trilux counter

(PerkinElmer).

These assays will indicate that [^{125}I]-ezetimibe binds to the cell

membranes expressing human, mouse or rat NPC1L1. Similar results will be

20   obtained if the same experiment is performed with radiolabeled cholesterol (*e.g.*,

^{125}I-cholesterol).

Example 3A: Alternate SPA Assay.

The final concentrations should be: 1 nM ^{35}S-**2** (Km ~ 2-5 nM,

~50,000 dpm/assay);

25   1 μg membranes (~1-2 nM receptor); 0.007%-0.03% taurocholate

(0.140 μl 1% stock); 0.010%-0.05% digitonin (0.200 μl 1% stock); 5% DMSO (1.00

μl inhibitors).

In each well of a 96 well plate is put 1 μl DMSO inhibitor solution, and

then the radioligand and detergents are added as a 2X solution in 10 μl buffer A.

30   Shake for a minute to be sure the inhibitor and ligand are mixed, before initiating with

9 μl diluted receptor solution in buffer A. After shaking again, the plate is incubated

at 37°C for 2 hours. Then WGA beads (0.3 mg) are added as a 3 μl suspension in

buffer A, then shake for 30 minutes.   Similar results are obtained if membranes are
pre-incubated with beads for 30 minutes before adding ligands. Finally, dilute to
300 µl with buffer A, cover the plate, spin at 3,000 rpmx5 min, and read at 2 minutes
per well in the "Microbeta" counter.

5 **Stocks**

Ligand: [35]S-2 is 525.42 nM, 0616 µCi/µl, in acetonitrile; spec. act.
$= 3.8916 \times 10^{-4}$ fmol/dpm; 1168 Ci/mmol)

Membranes: 3rd batch recombinant human expressed in
HEK-293 cells; 20.2 ug/µl stock; ~20–40 pmol NPC1L1/mg protein

10   Buffer A: 26 mM $NaHCO_3$; 0.96 mN NaH_2PO_4; 5 mM HEPES;
optional addition of 5.5 mM glucose; 117 mM NaCl; 5.4 mM KCl

Example 4: Cholesterol Uptake Assay.

CHO cells expressing either SR-B1 or three different clones of rat
NPC1L1 or one clone of mouse NPC1 L1 were starved overnight in cholesterol free
15 media then dosed with [3H]-cholesterol in a mixed synthetic micelle emulsion for 4
min, 8 min, 12 min or 24 min in the absence or presence of 10 □M ezetimibe. The
cells were harvested and the lipids were organically extracted. The extracted lipids
were spotted on thin-layer chromatography (TLC) plates and resolved within an
organic vapor phase. The free cholesterol bands for each assay were isolated and
20 counted in a scintillation counter.

The SR-B1 expressing cells exhibited an increase in [3H]-cholesterol
uptake as early as 4 min which was also inhibited by ezetimibe. The three rat clones
and the one mouse clone appeared to give background levels of [3H]-cholesterol
uptake which was similar to that of the untransformed CHO cell.

25   These experiments will yield data demonstrating that CHO cells can
perform mouse, rat and human NPC1L1-dependent uptake of [3H]-cholesterol when
more optimal experimental conditions are developed.

Example 5: Expression of Rat NPC1L1 in Wistar Rat Tissue.

In these experiments, the expression of rat *NPC1L1* mRNA, in several
30 rat tissues, was evaluated. The tissues evaluated were esophagus, stomach,
duodenum, jejunum, ileum, proximal colon, distal colon, liver, pancreas, heart,
aorta, spleen, lung, kidney, brain, muscle, testes, ovary, uterus, adrenal gland and

thyroid gland. Total RNA samples were isolated from at least 3 male and 3 female
animals and pooled. The samples were then subjected to real time quantitative
PCR using Taqman analysis using standard dual-labeled fluorogenic
oligonucleotide probes. Typical probe design incorporated a 5' reporter dye

5   (e.g., 6FAM (6-carboxyfluorescein) or VIC) and a 3' quenching dye (e.g., TAMRA
(6-carboxytetramethyl-rhodamine)).

rat _NPC1L1_:

        Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 14)

        Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 15)

10   Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA]

(SEQ ID NO: 16) rat β-actin:

        Forward: ATCGCTGACAGGATGCAGAAG (SEQ ID NO: 17)

        Reverse: TCAGGAGGAGCAATGATCTTGA (SEQ ID NO: 18)

        Probe: [VIC]AGATTACTGCCCTGGCTCCTAGCACCAT[TAMRA]

15   (SEQ ID NO: 19)

        PCR reactions were run in 96-well format with 25 µl reaction mixture
in each well containing: Platinum SuperMix (12.5 µl), ROX Reference Dye (0.5 µl),
50 mM magnesium chloride (2 µl), cDNA from RT reaction (0.2 µl). Multiplex
reactions contained gene specific primers at 200 nM each and FAM labeled probe at

20   100 nM and gene specific primers at 100 nM each and VIC labeled probe at 50 nM.
Reactions were run with a standard 2-step cycling program, 95°C for 15 sec and 60°C
for 1 min, for 40 cycles.

        The highest levels of expression were observed in the duodenum,
jejunum and ileum tissue. These data indicate that NPC1L1 plays a role in cholesterol

25   absorption in the intestine.

Example 6: Expression of Mouse NPC1L1 in Mouse Tissue.

        In these experiments, the expression of mouse _NPC1L1_ mRNA, in
several tissues, was evaluated. The tissues evaluated were adrenal gland, BM, brain,
heart, islets of langerhans, LI, small intestine, kidney, liver, lung, MLN, PLN, muscle,

30   ovary, pituitary gland, placenta, Peyers Patch, skin, spleen, stomach, testes, thymus,
thyroid gland, uterus and trachea. Total RNA samples were isolate from at least 3
male and 3 female animals and pooled. The samples were then subjected to real time
quantitative PCR using Taqman analysis using the following primers and probes:

-58-

mouse *NPC1L1*:

        Forward: ATCCTCATCCTGGGCTTTGC (SEQ ID NO: 20)

        Reverse: GCAAGGTGATCAGGAGGTTGA (SEQ ID NO: 21)

        Probe: [6FAM]CCCAGCTTATCCAGATTTTCTTCTTCCGC[TAMRA]

5   (SEQ ID NO: 22)

        The highest levels of expression were observed in the Peyer's Patch, small intestine, gall bladder and stomach tissue. These data are consistent with a cholesterol absorption role for NPC1L1 which takes place in the digestive system.

Example 7: Expression of Human NPC1L1 in Human Tissue.

10   In these experiments, the expression level of human NPC1L1 mRNA was evaluated in 2045 samples representing 46 normal tissues. Microarray-based gene expression analysis was performed on the Affymetrix HG-U95 GeneChip using a cRNA probe corresponding to base pairs 4192-51 17 (SEQ ID NO: 43) in strict accordance to Affymetrix's established protocols. Gene Chips were scanned under

15   low photo multiplier tube (PMT), and data were normalized using either Affymetrix MAS 4.0 or MAS 5.0 algorithms. In addition "spike ins" for most samples were used to construct a standard curve and obtain RNA concentration values according Gene Logic algorithms and procedures. A summary of these results are indicated, below, in Table 2.

Table 2. Expression level of *NPC1L1* mRNA in various human tissues.

Tissue	Present	Absent	Lower 25%	Median	Upper 75%	Tissue	Present	Absent	Lower 25%	Median	Upper 75%
Adipose	2 of 32	30 of 32	-2.45	1.16	12.23	Liver	32 of 34	2 of 34	325.74	427.77	540.1
Adrenal Gland	0 of 12	12 of 12	-23.54	-4.47	10.51	Lung	2 of 93	91 of 93	-3.47	11.03	22.34
Appendix	0 of 3	3 of 3	-8.02	-6.69	38.19	Lymph Node	0 of 11	11 of 11	-1.78	-0.19	1.34
Artery	0 of 3	3 of 3	-6.59	-4.67	9.68	Muscles	0 of 39	39 of 39	-21.57	8.25	26.73
Bladder	1 of 5	4 of 5	-22	-7.95	-1.99	Myometrium	8 of 106	98 of 106	-3.98	4.87	17.55
Bone	0 of 3	3 of 3	-1.64	3.3	19.53	Omentum	0 of 15	15 of 15	-14.25	-1.6	19.58
Breast	4 of 80	76 of 80	-4.07	3.13	14.67	Ovary	1 of 74	73 of 74	0.5	17.51	38.28
Cerebellum	0 of 5	5 of 5	-3.04	3.24	15.38	Pancreas	0 of 34	34 of 34	-87.08	-53.2	-24.14
Cervix	3 of 101	98 of 101	-7.56	-0.07	20.69	Placenta	0 of 5	5 of 5	-20.4	-3.44	18.91
Colon	9 of 151	142 of 151	-10.19	0.31	18.36	Prostate	0 of 32	32 of 32	1.08	15.56	27.24
Cortex Frontal Lobe	0 of 7	7 of 7	1.4	8.46	11.75	Rectum	1 of 43	42 of 43	-9.26	-1.49	9.8
Cortex Temporal Lobe	0 of 3	3 of 3	7.1	8.5	15.87	Right Atrium	4 of 169	165 of 169	-19.32	-6.58	7.72
Duodenum	59 of 61	2 of 61	519.23	827.43	1101.67	Right Ventricle	1 of 160	159 of 160	-24.01	-6.49	10.06
Endometrium	0 of 21	21 of 21	-14.43	-8.39	2.79	Skin	0 of 59	59 of 59	-12.68	1.5	22.77
Esophagus	1 of 27	26 of 27	-10.93	-4.97	12.48	Small Intestine	46 of 68	22 of 68	21.21	493.93	939.2
Fallopian Tube	3 of 51	48 of 51	5.02	13.24	26.77	Soft Tissues	1 of 6	5 of 6	-1.99	2.6	5.32
GallBladder	0 of 0	0 of 8	205.76	273.39	422.8	Spleen	0 of 31	31 of 31	-9.41	-0.31	9.5
Heart	0 of 3	3 of 3	3.33	11.19	11.66	Stomach	7 of 47	40 of 47	19.02	52.29	117.09
Hippocampus	0 of 5	5 of 5	8.26	9.11	19.83	Testis	0 of 5	5 of 5	-4.51	1.22	11.2
Kidney	4 of 86	82 of 86	-8.36	3.41	16.46	Thymus	1 of 71	70 of 71	-6.26	2.51	11.67
Larynx	0 of 4	4 of 4	-13.76	-0.81	8.54	Thyroid Gland	1 of 18	17 of 18	-12.22	2.84	17.86
Left Atrium	2 of 141	139 of 141	-18.9	-4.58	6.84	Uterus	0 of 58	58 of 58	-10.67	1.59	16.01
Left Ventricle	0 of 15	15 of 15	-21.19	-9.59	17.7	WBC	3 of 40	37 of 40	-16.45	-0.72	25.18

Shaded data corresponds to tissues wherein the highest levels of
NPC1L1 mRNA was detected. The "Present" column indicates the proportion of
specified tissue samples evaluated wherein *NPC1L1* mRNA was detected. The
5   "Absent" column indicates the proportion of specified tissue samples evaluated
wherein *NPC1L1* RNA was not detected. The "lower 25%", "median" and "upper
75%" columns indicate statistical distribution of the relative NPC1L1 signal
intensities observed for each set of tissue evaluated.

Example 8: Distribution of Rat NPC1L1, Rat IBAT or
10   **Rat SR-B1 mRNA in Rat Small Intestine.**

In these experiments, the distribution of rat *NPC1L1* mRNA along the
proximal-distal axis of rat small intestines was evaluated. Intestines were isolated
from five independent animals and divided into 10 sections of approximately equal
length. Total RNA was isolated and analyzed, by real time quantitative PCR using
15   Taqman analysis, for localized expression levels of rat *NPC1L1*, rat *IBAT* (ileal bile
acid transporter) or rat *SR-B1* mRNA. The primers and probes used in the analysis
were:

rat *NPC1L1*:

    Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 23)

    Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 24)

    Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA]

5 (SEQ ID NO: 25) rat Villin:

    Forward: AGCACCTGTCCACTGAAGATTTC (SEQ ID NO: 26)

    Reverse: TGGACGCTGAGCTTCAGTTCT (SEQ ID NO: 27)

    Probe: [VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA] (SEQ

ID NO: 28) rat *SR*-B1:

10   Forward: AGTAAAAAGGGCTCGCAGGAT (SEQ ID NO: 29)

    Reverse: GGCAGCTGGTGACATCAGAGA (SEQ ID NO: 30)

    Probe: [6FAM]AGGAGGCCATGCAGGCCTACTCTGA[TAMRA]

(SEQ ID NO: 31) rat *IBAT*:

    Forward: GAGTCCACGGTCAGTCCATGT (SEQ ID NO: 32)

15   Reverse: TTATGAACAACAATGCCAAGCAA (SEQ ID NO: 33)

    Probe: [6FAM]AGTCCTTAGGTAGTGGCTTAGTCCCTGGAAGC

TC[TAMRA] (SEQ ID NO: 34)

    The mRNA expression levels of each animal intestinal section were

analyzed separately, then the observed expression level was normalized to the

20 observed level of villin mRNA in that intestinal section. The observed, normalized

mRNA expression levels for each section where then averaged.

    The expression level of *NPC1L1* and *SR-B1* were highest in the

jejunum (sections 2-5) as compared to that of the more distal ileum sections. Since

the jejunum is believed to be the site of cholesterol absorption, these data suggest

25 such a role for rat NPC1L1. *IBAT* distribution favoring the ileum is well document

and served as a control for the experiment.

Example 9: In situ Analysis of Rat NPC1L1 mRNA in Rat Jejunum Tissue.

    The localization of rat *NPC1L1* mRNA was characterized by *in situ*

hybridization analysis of rat jejunum serial sections. The probes used in this analysis

30 were:

    T7-sense probe: GTAATACGACTCACTATAGGGCCCTGACGGT

CCTTCCTGAGGGAATCTTCAC (SEQ ID NO: 35)

<u>T7-antisense probe</u>: GTAATACGACTCACTATAGGGCCTGGGAA

GTTGGTCATGGCCACTCCAGC (SEQ ID NO: 36)

The RNA probes were synthesized using T7 RNA polymerase

amplification of a PCR amplified DNA fragment corresponding rat *NPC1L1*

5   nucleotides 3318 to 3672 (SEQ ID NO 1). Sense and anti-sense digoxigenin–UTP

labeled cRNA probes were generated from the T7 promoter using the DIG RNA

Labeling Kit following the manufacturer's instructions. Serial cryosections rat

jejunum were hybridized with the sense and antiisense probes. Digoxigenin labeling

was detected with the DIG Nucleic Acid Detection Kit based on previous methods. A

10   positive signal is characterized by the deposition of a red reaction product at the site

of hybridization.

The anti-sense probe showed strong staining of epithelium along the

crypt-villus axis under low magnification (40X). The observed rat *NPC1L1* mRNA

expression levels may have been somewhat greater in the crypts than in the villus tips.

15   Under high magnification (200X), staining was observed in the enterocytes but not in

the goblet cells. A lack of staining observed with the sense probe (control) confirmed

the high specificity of the *NPC1L1* anti-sense signal. These data provided further

evidence of the role of rat NPC1L1 in intestinal cholesterol absorption.

Example 10: FACS Analysis of Fluorescently Labeled
20   **Ezetimibe Binding to Transiently Transfected CHO Cells.**

In these experiments, the ability of BODIPY-labeled ezetimibe

(Altmann, *et al.,* (2002) Biochim. Biophys. Acta 1580(1): 77-93) to bind to *NPC1L1*

and *SR-B1* was evaluated. "BODIPY" is a fluorescent group which was used to

detect the BODIPY-ezetimibe. Chinese hamster ovary (CHO) cells were transiently

25   transfected with rat *NPC1L1* DNA (rNPC1L1/CHO), mouse *NPC1L1* DNA

(mNPC1L1/CHO), mouse *SR-B1* DNA (mSRB1/CHO) or *EGFP* DNA (EGFP/CHO).

EGFP is enhanced green fluorescent protein which was used as a positive control.

The transfected CHO cells or untransfected CHO cells were then stained with 100 nM

BODIPY-labeled ezetimibe and analyzed by FACS. Control experiments were also

30   performed wherein the cells were not labeled with the BODIPY-ezetimibe and

wherein untransfected CHO cells were labeled with the BODIPY-ezetimibe.

No staining was observed in the untransfected CHO, rNPC1L1/CHO

or mNPC1L1/CHO cells. Fluorescence was detected in the positive-control

EGFP/CHO cells. Staining was also detected in the mouse SR-B1/CHO cells. These data show that, under the conditions tested, BODIPY-ezetimibe is capable of binding to SR-B1 and that such binding is not ablated by the presence of the fluorescent BODIPY group. When more optimal conditions are determined, BODIPY-ezetimibe

5   will be shown to label the rNPC1L1/CHO and mNPC1L1/CHO cells.

Example 11: FACS Analysis of Transiently Transfected CHO Cells Labeled with Anti-FLAG Antibody M2.

In these experiments, the expression of FLAG-tagged NPC1L1 on CHO cells was evaluated. CHO cells were transiently transfected with mouse

10   *NPC1L1* DNA, rat *NPC1L1* DNA, FLAG- rat *NPC1L1* DNA or FLAG- mouse *NPC1L1* DNA. The 8 amino acid FLAG tag used was DYKDDDDK (SEQ ID NO: 37) which was inserted on the amino-terminal extracellular loop just past the secretion signal sequence. The cells were incubated with commercially available anti-FLAG monoclonal mouse antibody M2 followed by a BODIPY-tagged anti-mouse

15   secondary antibody. The treated cells were then analyzed by FACS.

The M2 antibody stained the CHO cells transfected with FLAG-rat *NPC1L1* DNA and with FLAG–mouse NPC1L1. No staining was observed in the CHO cells transfected with mouse *NPC1L1* DNA and with rat *NPC1L1* DNA. These data showed that rat NPC1L1 and mouse NPC1L1 possess no significant, inherent

20   fluorescence and are not bound by the anti-FLAG antibody. The observed, FLAG-dependent labeling of the cells indicated that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are localized at the cell membrane of the CHO cells.

Example 12: FACS Analysis of FLAG-rat NPC1L1-EGFP Chimera in Transiently Transfected CHO Cells.

25   In these experiments, the surface and cytoplasmic localization of rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG- rat *NPC1L1* DNA or with FLAG-rat *NPC1L1-EGFP* DNA. In these fusions, the FLAG tag is at amino-terminus of rat NPC1L1 and EGFP fusion is at the carboxy-terminus of rat NPC1L1. The cells were then stained with the M2 anti-FLAG mouse

30   (primary) antibody followed by secondary staining with a BODIPY-labeled anti-mouse antibody. In control experiments, cells were stained with only the secondary antibody and not with the primary antibody (M2). The stained cells were then analyzed by FACS.

In a control experiment, FLAG-rat NPC1L1 transfected cells were
stained with BODIPY anti-mouse secondary antibody but not with the primary
antibody. The data demonstrated that the secondary, anti-mouse antibody possessed
no significant specificity for FLAG-rat NPC1L1 and that the FLAG-rat NPC1L1,
5   itself, possesses no significant fluorescence.

        In another control experiment, unlabeled FLAG-rat NPC1L1-EGFP
cells were FACS analyzed. In these experiments, autofluorescence of the enhanced
green fluorescent protein (EGFP) was detected.

        FLAG-rat NPC1L1 cells were stained with anti-FLAG mouse antibody
10   M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS
analyzed. The data from this analysis showed that the cells were labeled with the
secondary, BODIPY-labeled antibody which indicated expression of the FLAG-rat
NPC1L1 protein on the surface of the CHO cells.

        FLAG-rat NPC1L1-EGFP cells were stained with anti-FLAG mouse
15   antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and
FACS analyzed. The data from this analysis showed that both markers (BODIPY and
EGFP) were present indicating surface expression of the chimeric protein. The data
also indicated that a portion of the protein was located within the cells and may be
associated with transport vesicles. These data supported a role for rat NPC1L1 in
20   vesicular transport of cholesterol or protein expressed in subcellular organelles such
as the rough endoplasmic reticulum.

**Example 13: FACS Analysis and Fluorescent Microscopy of
FLAG-rat NPC1L1-EGFP Chimera in a Cloned CHO Cell Line.**

        In these experiments, the cellular localization of rat NPC1L1 was
25   evaluated by FACS analysis and by immunohistochemistry. CHO cells were
transfected with FLAG-rat *NPC1L1-EGFP* DNA and stained with anti-FLAG mouse
antibody M2 and then with a BODIPY-labeled anti-mouse secondary antibody. In the
fusion, the FLAG tag is at the amino-terminus of rat NPC1L1 and the enhanced green
fluorescent protein (EGFP) tag is located at the carboxy-terminus of the rat NPC1L1.
30   The stained cells were then analyzed by FACS and by fluorescence microscopy.

        Cells transfected with FLAG—rat *NPC1L1-EGFP* DNA were stained
with the anti-FLAG mouse antibody M2 and then with the BODIPY-labeled anti-

mouse secondary antibody. FACS analysis of the cells detected both markers

indicating surface expression of the chimeric protein.

FLAG–rat NPC1L1-EGFP transfected cells were analyzed by

fluorescent microscopy at 63X magnification. Fluorescent microscopic analysis of

5   the cells indicated non-nuclear staining with significant perinuclear organelle staining.

Resolution of the image could not confirm the presence of vesicular associated

protein. These data indicated that the fusion protein was expressed on the cell

membrane of CHO cells.

Example 14: Generation of Polyclonal Anti-rat NPC1L1 Rabbit Antibodies.

10   Synthetic peptides (SEQ ID NO: 39-42) containing an amino- or

carboxy-terminal cysteine residue were coupled to keyhole limpet hemocyanin (KLH)

carrier protein through a disulfide linkage and used as antigen to raise polyclonal

antiserum in New Zealand white rabbits (range 3-9 months in age). The KLH-peptide

was emulsified by mixing with an equal volume of Freund's Adjuvant, and injected

15   into three subcutaneous dorsal sites. Prior to the 16 week immunization schedule a

pre-immune sera sample was collected which was followed by a primary injection of

0.25 mg KLH-peptide and 3 scheduled booster injections of 0.1 mg KLH-peptide.

Animals were bled from the auricular artery and the blood was allowed to clot and the

serum was then collected by centrifugation

20   The anti-peptide antibody titer was determined with an enzyme linked

immunosorbent assay (ELISA) with free peptide bound in solid phase (1 µg/well).

Results are expressed as the reciprocal of the serum dilution that resulted in an OD_{450}

of 0.2. Detection was obtained using the biotinylated anti-rabbit IgG, horse radish

peroxidase–streptavidin (HRP-SA) conjugate, and ABTS.

25   **Example 15: FACS Analysis of Rat NPC1L1 Expression in
CHO Cells Transiently Transfected with Rat NPC1L1 DNA
Using Rabbit Anti-rat NPC1L1 Antisera.**

In these experiments, the expression of rat NPC1L1 on the surface of

CHO cells was evaluated. CHO cells were transfected with rat *NPC1L1* DNA, then

30   incubated with either rabbit preimmune serum or with 10 week anti-rat NPC1L1

serum described, above, in Example 14 (i.e., A0715, A0716, A0867 or A0868). Cells

labeled with primary antisera were then stained with a BODIPY-modified anti-rabbit

secondary antibody followed by FACS analysis.

No antibody surface labeling was observed for any of the pre-immune sera samples. Specific cell surface labeling of rat NPC1L1 transfected cells was observed for both A0715 and A0868. Antisera A0716 and A0867 did not recognize rat NPC1L1 surface expression in this assay format. This indicates that the native,

5   unfused rat NPC1L1 protein is expressed in the CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 16: FACS Analysis of CHO Cells Transiently Transfected with FLAG-Mouse NPC1L1 DNA or FLAG-rat NPC1L1 DNA or
10   **Untransfected CHO Cells Using Rabbit Anti-rat NPC1L1 Antisera.**

In these experiments, the expression of FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG-mouse *NPC1L1* DNA or with FLAG-rat *NPC1L1* DNA. The FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 transfected cells were labeled with

15   either A0801, A0802, A0715 or A0868 sera (see Example 14) or with anti-FLAG antibody, M2. The labeled cells were then stained with BODIPY-labeled anti-rabbit secondary antibody and FACS analyzed. The untransfected CHO cells were analyzed in the same manner as the transfected cell lines.

Positive staining of the untransfected CHO cells was not observed for
20   any of the antisera tested. Serum A0801-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed but such labeling of FLAG-mouse NPC1L1 transfected cells was not observed. Serum A0802-dependent labeling of FLAG-mouse NPC1L1 or FLAG-rat NPC1L1 transfected cells was not observed. Strong serum A0715-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed and weak

25   serum A0715-dependent labeling of FLAG-mouse NPC1L1 transfected cells was observed. Weak serum A0868-dependent labeling of rat NPC1L1 and mouse NPC1L1 transfected cells was observed. Strong Anti-FLAG M2 antibody-dependent labeling of FLAG-rat NPC1L1 and FLAG-mouse NPC1L1 transfected cells was observed. The strong M2 staining is likely to be due to the fact that M2 is an affinity-

30   purified, monoclonal antibody of known concentration. In contrast, the respective antisera are polyclonal, unpurified and contain an uncertain concentration of anti-rat NPC1L1 antibody. These date provide further evidence that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are expressed in CHO cells and localized to

the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 17: Immunohistochemical Analysis of Rat Jejunum Tissue with Rabbit Anti-rat NPC1L1 Antisera A0715.

5   In these experiments, the localization of rat NPC1L1 in rat jejunum was analyzed by immunohistochemistry. Rat jejunum was removed, immediately embedded in O.C.T. compound and frozen in liquid nitrogen. Sections (6μm) were cut with a cryostat microtome and mounted on glass slides. Sections were air dried at room temperature and then fixed in Bouin's fixative. Streptavidin-biotin-peroxidase

10 immunostaining was carried out using Histostain-SP kit. Endogenous tissue peroxidase activity was blocked with a 10 minute incubation in 3% H_2O_2 in methanol, and nonspecific antibody binding was minimized by a 45 minute incubation in 10% nonimmune rabbit serum. Sections were incubated with a rabbit anti-rat NPC1L1 antisera A0715 or A0868 at a 1: 500 dilution at 4°C, followed by incubation with

15 biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Subsequently, the sections were developed in an aminoethyl carbazole (AEC)-H_2O_2 staining system and counterstained with hematoxylin and examined by microscopy. A positive reaction using this protocol is characterized by the deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin

20 counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum, (3) substitute the primary antibody with PBS, (4) substitute the second antibody with PBS.

25   The example shows tissue stained with anti-rat NPC1L1 sera A0715 or with the preimmune sera analyzed at low magnification (40X) and at high magnification (200X). The A0715-stained tissue, at low magnification, showed positive, strong staining of the villi epithelial layer (enterocytes). The A0715-stained tissue at high magnification showed positive, strong staining of the enterocyte apical

30 membranes. No staining was observed in tissue treated only with preimmune sera. Similar results were obtained with sera A0868. These data indicate that rat NPC1L1 is expressed in rat jejunum which is consistent with a role in intestinal cholesterol absorption.

Example 18: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells stably transfected with rat NPC1L1 to take up labeled cholesterol was evaluated. In these assays, cholesterol uptake, at a single concentration, was evaluated in a pulse-chase experiment. The data generated in these experiments are set forth, below, in Table 3.

Cells:

A.   CHO cells stably transfected with rat *NPC1L1* cDNA

B.   CHO background (no transfection)

Cells were seeded at 500,000 cells/ well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS).

One plate of each cell line was starved overnight. The remaining 2 plates were designated "No Starve" (see below).

Pre-Incubation. Media was removed from all plates, rinsed with serum-free HAMS and replaced with starve media for 30 minutes.

^{3}**H-Cholesterol Pulse.** The following was added directly to each well.

0.5µCi ^{3}H-cholesterol (~1.1 X 10^6 dpm/well) in 50µl of a mixed bile salt micelle.

4.8 mM sodium taurocholate (2.581 mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5µg/mL

Labeled cholesterol pulse time points were 0, 4, 12 and 24 minutes. Triplicate wells for each treatment were prepared.

Wash. At the designated times, media was aspirated and the cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis. Cells were digested overnight with 0.2N NaOH, 2mL/well at room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting. Two additional 50µl aliquots from all wells are assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized by the quantity of protein in the cells.

Table 3. Uptake of 3H-cholesterol by CHO cells transfected with rat *NPC1L1* or mouse *SR-B1* or untransfected CHO cells.

Time, min After ^{3}H-Cholesterol	Total Cholesterol, dpm protein ± sem				Total Cholesterol, dpm/mg protein ± sem			
	NPC1L1		CHO		NPC1L1		CHO	
				No Starve				
0	2067	±46	4568	±1937	10754	±166	22881	±9230
4	2619	±130	2868	±193	15366	±938	15636	±1471
12	2868	±193	4459	±170	15636	±1471	24622	±966
24	7010	±89	7204	±173	41129	±685	39361	±1207
				Starve				
0	1937	±273	2440	±299	10909	±1847	12429	±1673
4	3023	±308	2759	±105	17278	±1650	14307	±781
12	2759	±105	4857	±186	14307	±781	26270	±1473
24	6966	±72	7344	±65	39196	±174	38381	±161

dpm=disintegrations per minute
sem=standard error of the mean

Example 19: Effect of Ezetimibe on Cholesterol Uptake.

The effect of ezetimibe on the ability of CHO cells stably transfected with mouse or rat *NPC1L1* or mouse *SR-B1* to take up ^{3}H-labeled cholesterol was evaluated in pulse-chase experiments. One cDNA clone of mouse *NPC1L1* (C7) and three clones of rat *NPC1L1* (C7, C17 and C21) were evaluated. The ability of CHO

cells stably transfected with mouse *SR-B1*, mouse *NPC1L1* and rat *NPC1L1* to take up labeled cholesterol, in the absence of ezetimibe, was also evaluated in the pulse-chase experiments. Data generated in these experiments are set forth, below, in Tables 4 and 5. Additionally, the quantity of total cholesterol taken up by transfected and

5   untransfected CHO cells in the presence of four different unlabeled cholesterol concentrations was also evaluated. The data from these experiments is set forth, below, in Table 6.

Cells:

A.   CHO cells stably transfected with rat or mouse *NPC1L1* cDNA

10 B.   CHO background (no transfection)

C.   *SR-B1* transfected CHO cells

Cells seeded at 500,000 cells / well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless

15 otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). The cells were then starved overnight.

20   **Pre-Incubation/ pre-dose.** Media was removed from all plates and replaced with fresh starve media and preincubated for 30 minutes. Half of the wells received media containing ezetimibe (stock soln in EtOH; final conc. = 10μM).

[3]**H-Cholesterol Pulse.** The following was added directly to each well:

0.5μCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50μl of a

25 mixed bile salt micelle

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

30   Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 4, 12, 24 minutes and 4 hours. Triplicate wells were prepared for each treatment.

Wash. At designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% bovine serum albumin (BSA), pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

5   **Processing/Analysis.**

              A.   4, 12, 24 minute time points: Cells were digested overnight with 0.2N NaOH, 2mL/well, room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

              B.   4 hour time point: The digested cells were analyzed by thin-

10 layer chromatography to determine the content of cholesterol ester in the cells.

              Extracts were spotted onto TLC plates and run for 30 minutes in 2 ml hexane: isopropanol (3: 2) mobile phase for 30 minutes, followed by a second run in 1 ml hexane: isopropanol (3: 2) mobile phase for 15 minutes.

              C.   Protein determination of cell extracts. Plates containing a

15 sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12 X 75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50μl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was

20 normalized to the quantity of protein in the cells.

Table 4. Total Cholesterol in Transfected CHO Cells in the Presence and Absence of

Ezetimibe.

Clones:	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 µM)		Vehicle		EZ (10µM)	
4 Min Pulse								
CHO Control	3413	±417	3222	±26	33443	±4070	31881	±483
SR-BI	14207	±51	10968	±821	118242	±1261	92474	±2902
mNPC1L1(C7)	4043	±419	4569	±222	30169	±3242	30916	±1137
rNPC1L1(C21)	3283	±288	3769	±147	23728	±2111	27098	±689
rNPC1L1(C17)	3188	±232	3676	±134	24000	±832	28675	±527
rNPC1L1(C7)	1825	±806	3268	±121	15069	±6794	27285	±968
12 Min Pulse								
CHO Control	4710	±246	4532	±165	44208	±2702	43391	±1197
SR-BI	16970	±763	12349	±298	140105	±6523	98956	±4447
mNPC1L1(C7)	6316	±85	6120	±755	45133	±342	41712	±4054
rNPC1L1(C21)	5340	±12	4703	±231	40018	±1181	33985	±1928
rNPC1L1(C17)	4831	±431	4579	±257	37378	±3461	34063	±1619
rNPC1L1(C7)	4726	±272	4664	±63	39100	±2350	38581	±784
24 Min Pulse								
CHO Control	7367	±232	6678	±215	65843	±1281	61764	±2131
SR-BI	39166	±2152	23558	±1310	324126	±11848	198725	±11713
mNPC1L1(C7)	10616	±121	9749	±482	77222	±1040	74041	±3670
rNPC1L1(C21)	9940	±587	8760	±293	76356	±9618	66165	±2181
rNPC1L1(C17)	8728	±721	8192	±237	70509	±5189	62279	±4352
rNPC1L1(C7)	8537	±148	7829	±204	72134	±1305	63482	±368

EZ = ezetimibe

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Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

Clones:	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	Vehicle		EZ (10 µM)		Vehicle		EZ (10µM)	
	4 Hour Pulse							
CHO Control	652	±13	208	±9	5647	±55	1902	±87
SR-BI	47608	±1292	9305	±401	391067	±14391	72782	±3181
mNPC1L1(C7)	732	±127	453	±118	4994	±827	3057	±776
rNPC1L1(C21)	2667	±90	454	±33	18655	±1032	3193	±265
rNPC1L1(C17)	751	±74	202	±10	5379	±481	1510	±62
rNPC1L1(C7)	462	±25	191	±54	3597	±193	1496	±403

	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 µM)		Vehicle		EZ (10µM)	
	4 Hour Pulse							
CHO Control	61612	±1227	56792	±568	533876	±17770	519607	±16203
SR-BI	214678	±4241	194519	±474	1762873	±46607	1521341	±4185
mNPC1L1(C7)	79628	±793	77516	±1910	544661	±1269	523803	±10386
rNPC1L1(C21)	71352	±1343	69106	±711	498016	±8171	485460	±4410
rNPC1L1(C17)	78956	±3782	71646	±446	566456	±29204	536651	±7146
rNPC1L1(C7)	75348	±2093	70628	±212	586127	±13932	556855	±7481

EZ = ezetimibe

Table 6. Uptake of labeled cholesterol in the presence of increasing amounts of unlabeled cholesterol.

	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
Cold Cholesterol	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
24 Min Pulse								
3 µg/mL	12271 ±430	49603 ±2428	14250 ±1628	10656 ±1233	108936 ±5413	541562 ±13785	140764 ±14433	94945 ±12916
10 µg/mL	16282 ±2438	79967 ±8151	25465 ±3037	13225 ±4556	151283 ±23345	880224 ±82254	250985 ±27481	123433 ±34092
30 µg/mL	14758 ±1607	71925 ±3863	19001 ±1530	13218 ±1149	135109 ±12106	796236 ±18952	180436 ±12112	111522 ±6941
100 µg/mL	16458 ±1614	58185 ±4548	15973 ±1665	11560 ±1132	149559 ±17977	630143 ±3718	147717 ±8261	101328 ±7191

	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
4 Hour Pulse								
3 µg/mL	2737 ±114	39596 ±1241	1561 ±1	4015 ±47	22050 ±978	382641 ±5955	13684 ±217	32020 ±641
10 µg/mL	1646 ±76	17292 ±362	998 ±36	1866 ±33	13323 ±606	157914 ±3400	8917 ±467	14849 ±127
30 µg/mL	970 ±46	6642 ±153	537 ±82	970 ±9	7627 ±325	63547 ±1760	4885 ±748	7741 ±100
100 µg/mL	895 ±156	4777 ±27	405 ±7	777 ±16	7135 ±1230	45088 ±1526	3663 ±68	6005 ±198

	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
4 Hour Pulse								
3 µg/mL	89013 ±3724	211783 ±3268	104343 ±2112	92244 ±987	717308 ±34130	2047695 ±16213	914107 ±5869	735498 ±11209
10 µg/mL	136396 ±8566	278216 ±10901	196173 ±4721	125144 ±877	1105118 ±76074	2540130 ±92471	1753072 ±86578	996824 ±27850
30 µg/mL	131745 ±2922	224429 ±2556	149172 ±19689	117143 ±4976	1036195 ±21142	2149315 ±78068	1357136 ±180264	934772 ±43202
100 µg/mL	79336 ±4011	231470 ±4221	114599 ±2803	93538 ±1588	632965 ±29756	2182022 ±36793	1035979 ±30329	723225 ±21694

	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
24 Hour Pulse								
3 µg/mL	57373 ±2704	162296 ±1644	22986 ±940	59377 ±953	357629 ±14639	1248900 ±18565	160328 ±6565	401315 ±5557
10 µg/mL	33730 ±1296	112815 ±373	14836 ±552	31797 ±525	215004 ±5942	830231 ±12764	98594 ±4205	200451 ±5239
30 µg/mL	19193 ±100	58668 ±1413	8878 ±355	18963 ±380	122071 ±1271	446581 ±3472	59091 ±2697	119728 ±2131
100 µg/mL	16761 ±398	31280 ±1270	8784 ±946	14933 ±311	103235 ±1739	272796 ±13392	60670 ±4597	96215 ±1023

	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
24 Hour Pulse								
3 µg/mL	248985 ±4207	357819 ±4519	285610 ±5187	227244 ±1016	1552637 ±18954	2752957 ±24984	1993256 ±56968	1536023 ±10304
10 µg/mL	231208 ±8927	269822 ±5872	311777 ±8227	231666 ±6198	1477414 ±85954	1984473 ±18420	2069980 ±25517	1461157 ±58517
30 µg/mL	203566 ±6008	225273 ±5932	279604 ±6612	209372 ±3386	1294878 ±41819	1716066 ±52581	1859476 ±29507	1321730 ±5452
100 µg/mL	178424 ±2379	167082 ±2211	229832 ±4199	182678 ±7709	1099648 ±25160	1455799 ±9885	1599244 ±76938	1177546 ±51191

Example 20: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells transiently transfected with rat *NPC1L1* or mouse *SR-B1* to take up labeled cholesterol was evaluated. Also evaluated was the ability of rat *NPC1L1* to potentiate the ability of CHO cells transfected with mouse *SR-B1* to take up labeled cholesterol. In these assays,

cholesterol uptake, at a single concentration, was evaluated in pulse-chase experiments. The data generated in these experiments are set forth, below, in Table 7.

  Cells:

  A. CHO background cells (mock transfection).

5 B. CHO cells transiently transfected with mouse SR-B1.

  C. CHO transiently transfected with rat *NPC1L1* cDNAs (n=8 clones).

  Transiently transfected cells were seeded at 300,000 cells / well (mL) in 12-well plates.

10 **Procedure:**

  All reagents and culture plates were maintained at 37°C unless otherwise noted.

  Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed from the cells and replaced with 1 mL "starve" media (F12 HAMS,

15 Pen/Strep, 5% lipoprotein deficient serum (LPDS). Cells were starved for 1 hour.

  ^{3}**H-Cholesterol** Pulse. The following was added directly to each well.

  0.5µCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50µl of a mixed bile salt micelle.

  4.8mM sodium taurocholate (2.581mg/mL)

20 0.6 mM sodium oleate (0.183 mg/mL)

  0.25 mM cholesterol (0.1 mg/mL)

  Dispersed in "starve" media by ultrasonic vibration

  Final media cholesterol concentration = 5µg/mL

  Labeled cholesterol pulse time points were 24 Min and 4 hours.

25 Triplicate wells for each treatment.

  Wash. At the designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis.

30 A. 24 minute time point: Cells were digested overnight with 0.2N NaOH, 2mL/well at room temp. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

B.   4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

The extracts were spotted onto thin layer chromatography plates and run in 2 ml hexane: isopropanol (3: 2) containing mobile phase for 30 minutes, followed by a second run in 1 ml hexane: isopropanol (3: 2) containing mobile phase for 15 min.

C.   Protein determination of cell extracts: Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12X75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 7. Labeled cholesterol uptake in transiently transfected CHO cells.

	Total Cholesterol, ± sem	
	dpm	dpm/mg protein
Transfection	24 Min Pulse	
CHO Control (mock)	4721 ±436	49024 ±4328
SR-BI(Transient)	5842 ±82	59445 ±1099
NPC1L1 (Transient)	4092 ±377	47026 ±2658
SR-BI/NPC1L1 (trans)	3833 ±158	52132 ±3071

	Cholesteryl Ester, ± sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	2132 ±40	20497 ±640
SR-BI(Transient)	5918 ±237	51812 ±1417
NPC1L1 (Transient)	1944 ±93	19788 ±642
SR-BI/NPC1L1 (trans)	4747 ±39	58603 ±1156

	Free Cholesterol, ± sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	45729 ±328	439346 ±5389
SR-BI(Transient)	50820 ±2369	444551 ±9785
NPC1L1 (Transient)	39913 ±1211	406615 ±6820
SR-BI/NPC1L1 (trans)	37269 ±1225	459509 ±6195

Example 21: Expression of rat, mouse and human NPC1L1.

In this example, *NPC1L1* was introduced into cells and expressed. Species specific NPC1L1 expression constructs were cloned into the plasmid pCDNA3 using clone specific PCR primers to generate the ORF flanked by appropriate restriction sites compatible with the polylinker of the vector. For all three species of NPC1L1, small intestine total tissue RNA was used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo dT as the

template primer. The rat *NPC1L1* was cloned as an EcoRI fragment, human *NPC1L1* was cloned as a XbaI/NotI fragment and mouse *NPC1L1* was cloned as an EcoRI fragment. Forward and reverse strand sequencing of each clone was performed to confirm sequence integrity. Standard transient transfection procedures were used

5   with CHO cells. In a 6-well plate CHO cells were plated 1 day before transfection at a plating density of 2×10^5 cells/well. The following day, cells were incubated with 2 µg plasmid DNA and 6 µL Lipofectamine for 5 hours followed a fresh media change. Forty-eight hours later, cells were analyzed for NPC1L1 expression using anti-NPC1L1 antisera by either FACS or western blot. To establish stable long term

10   cell lines expressing NPC1L1, transfected CHO cells were selected in the presence of geneticin (G418, 0.8 mg/ml) as recommended by the manufacturer (Life Technologies). Following one month of selection in culture, the cell population was stained with anti-NPC1L1 antisera and sorted by FACS. Individual positive staining cells were cloned after isolation by limiting dilution and then maintained in selective

15   media containing geneticin (0.5 mg/ ml).

        Other cell types less susceptible to transfection procedures have been generated using adenoviral vector systems. This system used to express NPC1L1 is derived from Ad 5, a type C adenovirus. This recombinant replication-defective adenoviral vector is made defective through modifications of the E1, E2 and E4

20   regions. The vector also has additional modifications to the E3 region generally affecting the E3b region genes RIDa and RIDb. NPC1L1 expression was driven using the CMV promoter as an expression cassette substituted in the E3 region of the adenovirus. Rat and mouse NPC1L1 were amplified using clone specific primers flanked by restriction sites compatible with the adenovirus vector Adenovirus

25   infective particles were produced from 293-D22 cells in titers of 5×10^{10} P/mL. Viral lysates were used to infect cells resistant to standard transfection methodologies. In Caco2 cells, which are highly resistant to heterologous protein expression, adenovirus mediated expression of NPC1L1 has been shown by western blot analysis to persist at least 21 days post-infection.

30   **Example 22: NPC1L1 Knock-Out Transgenic Mouse.**

        NPC1L1 knockout mice were constructed via targeted mutagenesis. This methodology utilized a targeting construct designed to delete a specific region of the mouse *NPC1L1* gene. During the targeting process the *E. coli* lacZ reporter gene

was inserted under the control of the endogenous *NPC1L1* promoter. The region in

NPC1L1 (SEQ ID NO: 45) being deleted is from nucleotide 790 to nucleotide 998.

The targeting vector contains the LacZ-Neo cassette flanked by 1.9 kb 5' arm ending

with nucleotide 789 and a 3.2 kb 3' arm starting with nucleotide 999. Genomic DNA

5   from the recombinant embryonic stem cell line was assayed for homologous

recombination using PCR. Amplified DNA fragments were visualized by agarose gel

electrophoresis. The test PCRs employed a gene specific primer, which lies outside

of and adjacent to the targeting vector arm, paired with one of three primers specific

to the LacZ-*Neo* cassette sequence. For 5'.PCR reconfirmation, the *NPC1L1* specific

10   oligonucleotide ATGTTAGGTGAGTCTGAACCTACCC (SEQ ID NO: 46) and for

3'PCR reconfirmation the NPC1L1 specific oligonucleotide

GGATTGCATTTCCTTCAAGAAAGCC (SEQ ID NO: 47) were used. Genotyping

of the F2 mice was performed by multiplex PCR using the NPC1L1 specific forward

primer TATGGCTCTGCCCTCTGCAATGCTC (SEQ ID NO: 48) the LacZ-Neo

15   cassette specific forward primer TCAGCAGCCTCTGTTCCACATACACTTC (SEQ

ID NO: 49) in combination with the NPC1L1 gene specific reverse primer

GTTCCACAGGGTCTGTGGTGAGTTC (SEQ ID NO: 50) allowed for

determination of both the targeted and endogenous alleles. Analysis of the PCR

products by agarose gel electrophoresis distinguished the wild-type, heterozygote and

20   homozygote null mouse from each other.

Example 23: Acute Cholesterol Absorption in NPC1L1-Deficient Mice.

To determine whether NPC1L1 plays a role in cholesterol absorption,

NPC1L1 deficient mice were studied.

Mice deficient in *NPC1L1* (-/-) were generated by breeding

25   heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

Non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) were

weighed and grouped (n=2 -/- and n=4 +/+). All animals were gavaged (Feeding

needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis,

MO) containing 1μCi ^{14}C-cholesterol (New England Nuclear, [$^{4-14}$C] Cholesterol,

30   NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO). Two hours

later, blood was collected by heart puncture. The liver was removed, weighed, and

three samples were placed into 20 ml counting vials. Tissues were digested in 1 ml of

1N NaOH at 60°C overnight. The tissue digests were acidified by addition of 250μl

of 4N HCl prior to liquid scintillation counting (LSC). Plasma was isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100μl aliquots of plasma were taken for LSC.

5   Cholesterol absorption, evaluated by this acute technique and expressed as the total amount of radioactive cholesterol in the plasma and liver, demonstrated that the wild type mice (+/+) absorbed an average of 11,773 dpm and *NPC1L1* deficient mice absorbed 992 dpm of the 14C-cholesterol. These results indicate that the *NPC1L1* deficient mice have a 92% reduction in cholesterol absorption. These data confirm the role of NPC1L1 in intestinal cholesterol

10   absorption. Inhibition of NPC1L1-mediated cholesterol absorption, in a subject, by administering NPC1L1 antagonists, such as ezetimibe, to the subject, are a useful way to reduce serum cholesterol levels and the occurrence of atherosclerosis in the subject.

Example 24: Cholesterol Absorption in NPC1L1 (NPC3)
Knockout Mice (Fecal Ratio Method: Cholesterol/Sitostanol).

15   In this example, cholesterol absorption and the activity of ezetimibe was determined in the *NPC1L1* knockout mice (-/-), heterozygous mice (+/-), and age matched wild-type mice (+/+).

        Cholesterol absorption in the mice was determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta.

20   1580(1): 77-93 (2002)). Mice (n= 4-6/group) were fed a standard rodent chow diet and in some groups treated daily with a maximally effective dose of ezetimibe (10 mg/kg). Mice were gavaged with ^{14}C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2μCi) in 0.1ml corn oil. Feces were collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels were determined by combustion in a Packard

25   Oxidizer. The fraction of cholesterol absorbed, as evaluated by the fecal dual isotope technique, was similar in wild type (+/+) and heterozygous mice (+/-) fed a chow diet (heterozygous mice absorbed 46 ±5% and age matched wild type mice absorbed 51 ±3% of the dose of ^{14}C-cholesterol). The *NPC1L1* knockout mice (-/-) absorbed 15.6 ±0.4% of the ^{14}C-cholesterol, which was similar to the wild type mice treated with a

30   maximally effective dose of ezetimibe (16.1 ±0.3%), and reduced by 69% compared to wild type mice (p<0.001). In *NPC1L1* knockout treated with ezetimibe at 10 mg/kg/day, cholesterol absorption was similar to that seen in the untreated knockout mice (16.2 ±0.6% compared to 15.6% ±0.4%, respectively). Thus, the majority of

cholesterol absorption is dependent on the presence of NPC1L1 and the residual cholesterol absorption in mice lacking NPC1L1 is insensitive to ezetimibe treatment. These results indicate that NPC1L1 is involved in the small intestinal enterocyte uptake and absorption of cholesterol and is in the ezetimibe sensitive pathway.

5   **Example 25: Mouse Screening Assay (Acute Cholesterol Absorption).**

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Mice deficient in *NPC1L1* (-/-) are generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

10   In a first set of experiments, non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) are weighed and grouped (n=1 to 4 -/- and n=1 to 4 +/+). All animals are gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1 μCi ^{14}C-cholesterol (New England Nuclear, [$^{4-14}$C] Cholesterol, NEC-018) and 0.1 mg carrier cholesterol mass

15   (Sigma; St. Louis, MO).

In another set of experiments, 1 to 4 wild-type *NPC1L1* mice (+/+) are treated identically to the mice in the first set of experiments, above, except that the mice are additionally fed a sample to be tested for the presence of an NPC1L1 antagonist.

20   Two hours later, blood is collected from each mouse by heart puncture. The liver is removed, weighed, and three samples are placed into 20 ml counting vials. Tissues are digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests are acidified by addition of 250 μl of 4N HCl prior to liquid scintillation counting (LSC). Plasma is isolated by centrifugation at 10,000 rpm for 5 minutes in a

25   microfuge and duplicate 100 μl aliquots of plasma are taken for LSC.

Cholesterol absorption, evaluated by this acute technique is expressed as the total amount of radioactive cholesterol in the plasma and liver. The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol absorption (as measured by the above described methods) in the wild-type *NPC1L1*

30   mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

Example 26: Mouse Screening Assay
(Fecal Ratio Method: Cholesterol/Sitostanol).

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Cholesterol absorption in the mice is determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)).

Three groups of mice (n=1-6/group) are assembled. Two separate groups comprise wild-type *NPC1L1* mice (+/+) and one group comprises *NPC1L1* deficient mice (-/-).

Each group is fed a standard rodent chow diet and in some groups treated daily. Mice are gavaged with ^{14}C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2μCi) in 0.1ml corn oil. One group of mice, which comprise wild-type NPC1L1 mice (+/+) are further fed a sample to be tested for the presence of an NPC1L1 antagonist. Feces are collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels are determined by combustion in a Packard Oxidizer.

The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol and/or sitostanol absorption (as measured by the above described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol and/or sitostanol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

Example 27: Binding Analysis Using Brush Border Membrane Vesicles

The following screening assay may be used to identify the presence of an NPC1L1 ligand in a sample.

Materials. The following two compounds were synthesized for the binding assay described herein , ^{3}H-ezetimibe glucuronide **1** (34.5 Ci/mmol) and its ^{35}S-propargyl-sulfonamide derivative **2** (800-1100 Ci/mmol).
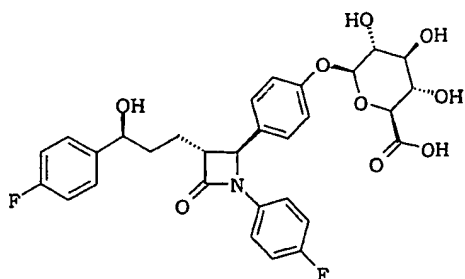
Ezetimibe-glucuronide
1
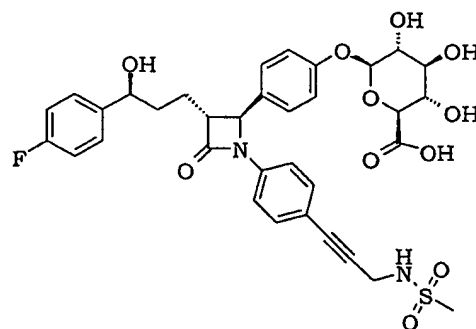
S-propargyl-sulfonamide
ezetimibe-glucuronide
2

Synthesis of ezetimibe glucuronide and S-propargyl-sulfonamide ezetimibe-glucuronide. Ezetimibe glucuronide (compound **1**) (also referred to as EZE-glucuronide) can be made according to the procedures in U.S. Patent No. 5,756,470. The general scheme below illustrates a method for the synthesis of compound **2** and radiolabel led ^{35}S-**2**.
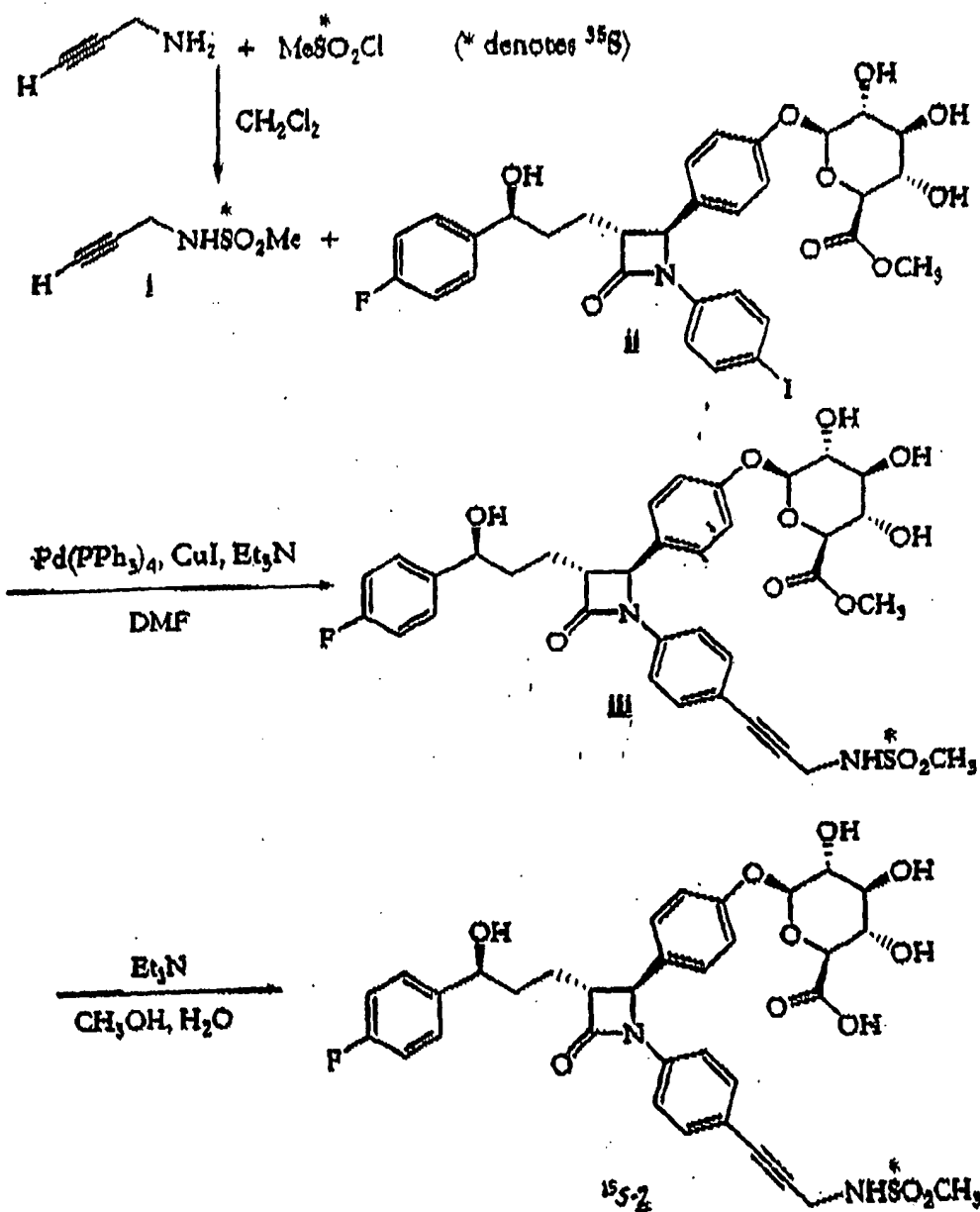
<u>Preparation of compound ^{35}S-2 (Compound 2 with radiolabelled ^{35}S)</u>

Step A: Preparation of [^{35}S]N-prop-2-yn-1-ylmethanesulfonamide (i).

The appropriate volume of [^{35}S]methane sulfonyl chloride (see Dean, D.C.; et al.,

J. Med. Chem. 1996, *39*, 1767) totaling 3.5 mCi was removed from a stock solution

5   in methylene chloride and placed in a 5mL conical flask. It was then distilled at

atmospheric pressure until the volume was approximately 50 μL. To this solution

was immediately added 50 μL of propargylamine. After 15 min, the reaction mixture

was diluted with 10 mL of ethyl acetate, washed with saturated sodium bicarbonate

solution (3 x 2 mL), and dried over sodium sulfate. After filtration the resulting

10   solution had a count of 3.3 mCi and a radiochemical purity of 99.9 % by HPLC

(Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H_2O (0.1 % TFA) to 100 %

acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 4.4 min).

Step B: Preparation of [^{35}S] 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-

hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-

15   oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate ([^{35}S]) (iii). Dissolved

3.0 mCi of [^{35}S]*N*-prop-2-yn-1-ylmethanesulfonamide, 1 mg of compound ii

(prepared according to Burnett, D.S. et al., Bioorg. Med. Chem. Lett. (2002), vol. 12,

p. 311), and 1 μL of triethylamine in 100 μL of dimethylformamide inside a plastic

microcentrifuge tube. To this solution was added 10 μL of a stock solution containing

20   8.1 mg of tetrakis(triphenylphosphine)palladium(0) and 1.4mg of copper iodide in 1

mL of dimethylformamide. Stirred at room temperature for sixty hours at which time

HPLC indicated 55% conversion. This reaction mixture, which had a radiochemical

purity of 44.4% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 %

acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1

25   mL/min, t_R = 9.3 min) was taken on directly to the next step.

Step C: Preparation of [^{35}S] 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-

hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-

oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid ^{35}S-2. The crude reaction

mixture containing compound iii was treated with 25 μL of methanol, 90 μL of water,

30   and 30 μL of triethylamine and stirred at room temperature for one hour at which time

it was concentrated to near dryness under a slow stream of nitrogen. The residue was

dissolved in 1:1 acetonitrile:H_2O and subjected to semi-preparative chromatography

(Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4

mL/min, 1 x 0.2 mL injections). 540 μCi of product was obtained which had a

radiochemical purity of 99.9% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm,

70:30 acetonitrile:H_2O (0.1 % TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an

5. authentic sample of compound **2**. LC/MS m/z = 508 (product – glucuronide – H_2O),

SA = 769 Ci/mmol.

Alternate Preparation of [35]S-**2**.

Step A: Preparation of **iii.** The appropriate volume of [^{35}S]methane

sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* **1996**, *39*, 1767) totaling 1.3

10   mCi was removed from a stock solution in methylene chloride and placed in a 5mL

conical flask. It was then distilled at atmospheric pressure until the volume was

approximately 50 μL. To this solution was immediately added a solution of 1 mg of **v**

in 5 μL of pyridine (freshly distilled over calcium hydride).

v

15
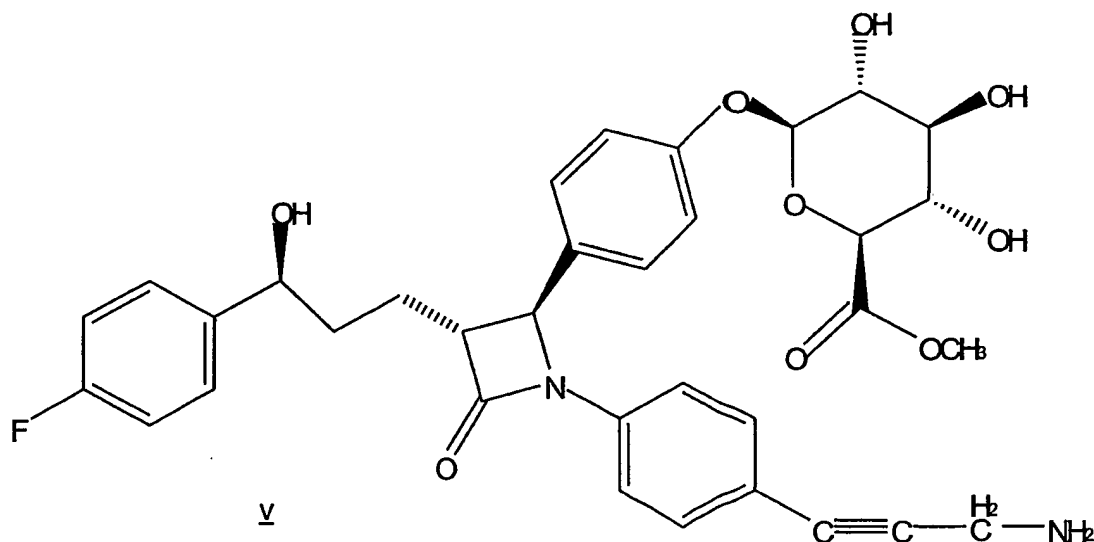
The solution was stirred at room temperature for five minutes at which

time it was concentrated to near dryness under a slow stream of nitrogen. This

reaction mixture, which had a radiochemical purity of 80.1% by HPLC (Zorbax XDB

C8 column, 4.6 x 150 mm, 5% acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15

20   min linear gradient, 1 mL/min, t_R = 9.3 min) was taken on directly to the next step.

Step B:Preparation of [35]S-**2**. The crude reaction mixture containing **iii**

was treated with 25 μL of methanol, 90 μL of water, and 30 μL of triethylamine and

stirred at room temperature for one hour at which time it was concentrated to near

dryness under a slow stream of nitrogen. The residue was dissolved in 1:1

acetonitrile:H_2O and subjected to semi-preparative chromatography (Zorbax XDB C8

250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL

5   injections). 350 µCi of product was obtained which had a radiochemical purity of 98.4

% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H_2O (0.1 %

TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an authentic sample of **2**. LC/MS

m/z = 508 (product – glucuronide – H_2O), SA = 911 Ci/mmol.

          Following the same general procedure for synthesis of ^{35}S-**2**, except

10   omitting the radiolabel ling, compounds **2** and **iv** can be prepared.

Preparation of brush border membrane vesicles (BBMV).

Membranes were prepared from Rhesus macaque (Macaca mulatta), rat (male

Sprague-Dawley), and mouse (male C57BL/6J) intestines, using Mg^{++} precipitation

method described in the following references and with modifications described below

15   (Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta

_602_, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal,

K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. _268_, 18035-18046 (1993);

Rigtrup, K.M., Ong, D.E. Biochemistry _31_, 2920-2926 (1992)).

          The intestines from freshly sacrificed animals were cut into segments,

20   perfused with ice-cold saline buffer (Buffer A: 26 mM $NaHCO_3$, 0.96 mM NaH_2PO_4,

5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4), placed on

cold glass plates, opened longitudinally, and the mucosa scraped with glass

microscope slips. This mucosa could be used fresh or frozen with identical results.

To prepare the membranes, the mucosal scrapings were resuspended in 20 volumes of

25   cold buffer consisting of 300 mM D-mannitol, 5 mM EGTA, 12 mM Tris, pH 7.4

with HCl, and containing 0.1 mM PMSF and a 1% dilution of a protease inhibitor

cocktail (set 1, Calbiochem). They were homogenized using a Polytron at medium

speed on ice until inspection with a microscope indicated complete cell lysis. Then,

solid $MgCl_2$ was added slowly with stirring to a final concentration of 10 mM, and the

30   solution was kept stirring on ice for 15 min. Cellular debris was removed by

centrifugation for 15 min at 3,000g, and the membranes were recovered by

centrifugation for 60 min at 48,000g. The membranes were further rinsed by re-

suspension in a buffer containing 50 mM D-mannitol, 5 mM EGTA, and 2 mM Tris

at pH 7.40, and centrifugation for 60 min at ≤48,000g. The final pellet was resuspended in 120 mM NaCl and 20 mM Tris at pH 7.40 to a concentration of ~10-20 mg protein/ml, aliquoted, frozen in liquid nitrogen, and stored at –80C. The activity was stable indefinitely and could be freeze-thawed with minimal loss of

5   activity.

Membrane protein was measured by the Bradford assay (Bradford, M.M. Anal. Biochem. 72, 248-254 (1976)) using bovine serum albumin as standard. The enrichment in brush border membranes was assessed using gamma-glutamyltransferase as a marker enzyme (Kramer, W., Girbig, F., Gutjahr, U.,

10   Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993)), which indicated a 6-fold enrichment over the initial homogenate.

Binding assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 ul. In general, frozen membranes were diluted in buffer A or

15   buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.02 to 5 mg/ml. Radiolabeled ligands were typically 25-50 nM for ^{3}H-ezetimibe (EZE)glucuronide **1**, and 3-5 nM for its ^{35}S analog **2**, in the assay, and they were delivered as DMSO or CH$_3$CN solutions. Competing ligands were likewise added as DMSO solutions to give a total 2-10 % organic solvent content. Nonspecific binding

20   was defined by competition with 100 uM ezetimibe glucuronide. At least 2 components of buffer A, the bicarbonate and phosphate salts, were later found to be inconsequential and were routinely omitted. To ensure equilibrium was established, the reactions with compound **1** were incubated at least 3 hours for rhesus membranes and at least one hour for rat membranes at room temperature, and with compound **2** as

25   long as 2 hours at 37°C with rhesus and rat brush membranes. Additionally, reactions with compound **2** were incubated as long as 2 hours at 37°C with membranes from HEK-293 cells expressing mouse, rat or human NPC1L1.

Bound ligand was quantified by single-tube vacuum filtration using GF/C glass fiber filters. Glass fiber filters (GF/C) were obtained from Whatman. The

30   filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70) to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with

another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Packard DM

liquid scintillation fluid. Where triplicate assays were performed, the standard error

was typically <4%. As an example, a 100μl assay of rat brush border membranes at 2

mg/ml in the presence of 400,000 dpm (50 nM) ^{3}H-ezetimibe glucuronide gave

15,000 dpm specific and 3,000 dpm nonspecific binding. The filters contributed most

of the nonspecific binding (2,000 dpm).

Alternatively, vacuum filtration of compound **2** on a Millipore 96-well

plate (Whatman GF/C) can also be used to achieve adequate precision.

Data analysis. Data from saturation experiments were subjected to a

Scatchard analysis, and linear regression was performed to yield the equilibrium

dissociation constant (K_d) and maximum receptor concentration (B_{max}). Correlation

coefficients for these determinations were typically greater than 0.96. Data from

competition experiments were analyzed and IC_{50} values determined from Hill plots of

the binding data. The kinetic data for ligand association and dissociation were

subjected to the analysis of Weiland and Molinoff (Weiland, G., Molinoff, .B. Life

Sci. 29, 313-330 (1981)). The dissociation rate constant for (k_{-1}) was determined

directly for a first order plot of ligand dissociation versus time. The rate of ligand

association (k_1) was determined from the equation $k_1 = k_{obs}([LR_e]/([L] [LR]_{max}))$

where [L] is the concentration of ligand, [LR_e] is the concentration of the complex at

·equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the

slope of the pseudo-first order plot $L_n ([LE]_e/([LR]_e - [LR]_t))$ versus time.

Binding analysis. Ezetimibe is rapidly converted to its glucuronide *in*

vivo, and this metabolite is thought to be largely if not exclusively responsible for

inhibition of cholesterol uptake. Accordingly, both ^{3}H-ezetimibe and its

corresponding glucuronide derivative (**1**) were prepared and tested for binding to

intestinal brush border membrane preparations, using a single-tube vacuum filtration

technique. As a result of the hydrophobic nature of ^{3}H-ezetimibe, high nonspecific

binding was observed, precluding its use as a radioligand for the binding assay.

However, due to the improved physical properties of the glucuronide derivative (**1**),

specific binding was observed with this radioligand and it was used to assess binding

in rhesus, rat, and mouse intestinal brush border membranes.

Rhesus, rat, and mouse intestinal scrapings were homogenized and the

brush border membranes isolated. Specific binding was observed exclusively in the

membrane fraction. Plots of total, nonspecific, and specific binding to rhesus (Figure 1) and rat (Figure 2) brush border membranes. Aliquots of rhesus BBMV (83 µg/assay) or rat BBMV (250µg/assay) were incubated with increasing concentrations of ^{3}H-EZE-glucuronide. Total binding and nonspecific binding determined in the

5   presence of 10-100 uM EZE-glucuronide are shown. Specific binding was calculated from the difference between total and nonspecific binding. Data were fit by nonlinear regression as described above, and the linear Scatchard plot is shown. In rhesus membranes, the data correspond to a single binding site with K_d = 41 nM and a concentration of 5.5 pmol/mg membrane protein. The affinity is ~10-fold lower in rat

10   membranes (K_d = 540 nM). ^{3}H-EZE-glucuronide is not the best ligand for a binding assay for the mouse target due to the compounds low affinity in mouse membrane. These potencies correlate roughly with the sensitivity of these species to ezetimibe inhibition of cholesterol uptake.

Rate constants for binding and dissociation. Ezetimibe-glucuronide

15   is slow to bind, and forms a relatively long-lived complex with its receptor. Indeed, this was key to detecting the interaction in a traditional filter-binding assay, as ligand/receptor interactions with K_d values greater than 100 nM often go unrecognized because of the typical fast off-rates of the ligands. Rate constants for association (k_{on}) and dissociation (k_{off}) of compound 1 were determined for rat and

20   rhesus membranes, and used as an alternative method to calculate the dissociation constant (K_d) according to the relationship $K_d = k_{off}/k_{on}$. 300µg/assay of rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide at room temperature for up to three hours for the association kinetic studies. 83 µg/assay of rhesus brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-

25   glucuronide at room temperature for up to five hours for the association kinetic studies. Nonspecific binding measured in the presence of 100µM EZE-glucuronide was subtracted from the total binding to calculate the specific binding shown in Figures 3A and 4A. For the dissociation kinetic study, rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide for 2 hours at room

30   temperature and ligand dissociation was initiated by the addition of 100µM EZE-glucuronide. Rhesus brush border membrane vesicles were incubated with 42nM ^{3}H-EZE-glucuronide for 4 hours at room temperature and ligand dissociation was initiated by the addition of 100µM EZE-glucuronide. For both rat and rhesus

dissociation studies, samples were collected at various times and radiolabel was detected. Dissociation curves are shown in Figures 3B (rat) and 4B (rhesus).

For rat membranes, the rate constant for association is $k_{on} = 5{,}540 \ M^{-1}$ s^{-1} (compared to 10^8 to $10^9 \ M^{-1} s^{-1}$ for diffusion controlled encounter), and the rate

5   constant for dissociation is $k_{off} = 2.4 \times 10^{-3} \ s^{-1}$, corresponding to a half-life of 4.7 min. The data are shown in Figure 3, where the solid lines are theoretical for these rate constants. The K_d value predicted from these rate constants ($K_d = k_{off}/k_{on} = 440 \ nM$) agrees well with that measured at equilibrium ($K_d = 540 \ nM$).

For rhesus membranes, where ^3H-ezetimibe glucuronide is at least

10   10-fold more potent (as described above), the association rate remains the same but the half-life for dissociation of the complex increases to ~90 min. These data are shown in Figure 4, where the theoretical lines correspond to $k_{on} = 3{,}900 \ M^{-1} s^{-1}$ and $k_{off} = 1.23 \times 10^{-4} \ s^{-1}$, and predict $K_d = 32 \ nM$ compared to that measured at equilibrium ($K_d = 41 \ nM$).

15   **Example 28: Binding Analysis of a Potent NPC1L1 ligand**

A ^{35}S-labeled propargyl-sulfonamide analogue of ezetimibe glucuronide (^{35}S-$\underline{2}$) was identified as a potential NPC1L1 antagonist. Compound $\underline{2}$ was prepared as described in Example 27 and found to have markedly improved affinity for some species of brush border membranes vesicles. For rhesus brush

20   border membranes vesicles, 56 μg protein/assay were incubated with 25 nM ^3H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and $\underline{2}$. For rat brush border membranes vesicles, 150 μg protein/assay were incubated with 50 nM ^3H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and $\underline{2}$. For mouse brush border membranes vesicles, 20 μg protein/assay

25   were incubated with 3 nM ^{35}S-$\underline{2}$ in the presence of increasing concentrations of EZE-glucuronide and $\underline{2}$.

$\underline{2}$ is more potent against enterocyte brush border membrane preparations from rats (35-fold), but is equipotent with ezetimibe glucuronide for rhesus membrane preparations (Figure 5, Table 8). It also has enhanced affinity for

30   mouse membranes (Figure 6, Table 8).

Table 8. Summary of inhibition constants (K$_i$) for binding of ezetimibe glucuronide **1** and its propargyl-sulfonamide derivative **2** to rhesus, rat, and mouse intestinal brush border membranes.

Compound	Rhesus	Rat	Mouse
1	39	530	2,300
2	38	15	144

5   K$_i$ values are nM.

Example 29: Distribution of ^{3}H-ezetimibe glucuronide (**1**) binding to intestinal tissues.

Previous studies have established that cholesterol absorption occurs primarily in the jejunum, and is substantially lower in the ileum and duodenum. To

10   determine if the binding activity is similarly distributed, the binding assay using ^{3}H-ezetimibe glucuronide (^{3}H-**1**) as a radioligand was used to determine the distribution of binding sites in sections from rhesus and rat intestines.

For the rhesus studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three

15   segments, (proximal, middle and distal) of equal length (70 cm each). For the rat studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (36 cm each). Brush border membrane vesicles were prepared as described in Example 27. Aliquots of vesicles (100-200μg) protein/assay were

20   incubated with 50nM ^{3}H-EZE-glucuronide in the absence and presence of 100μM EZE-glucuronide.

As shown in Figure 7, specific binding for ^{3}H-ezetimibe glucuronide peaks in the jejunum in both species, consistent with the previously observed pattern of cholesterol absorption.

25   **Example 30: Correlation of in vitro and in vivo binding activity of NPC1L1**

To determine if *in vitro* binding activity is predictive of *in vivo* efficacy, the enantiomer of ezetimibe glucuronide and several close structural analogues of ezetimibe glucuronide that were tested in the rat membrane binding assay were tested in an acute rat cholesterol absorption study as described in

30   Examples 23-26. The selected analogs had a range of *in vitro* potencies, and were

anticipated to have similar physical properties to ezetimibe glucuronide (Tables 9 and
12). The enantiomer, which has a Kd > 100,000 nM for the rat target, was inactive in
the *in vivo* assay. For the other analogs, the same rank order of potency is observed in
the *in vitro* and *in vivo* assays, further evidence that the observed binding is due to the
5   target of ezetimibe.

**Table 9. IC$_{50}$ values of EZE-gluc and analogs to inhibit binding of 3H-EZE-gluc
to rat brush border membrane vesicles.**

Compound Name	R^1	X	Y	RAT IC$_{50}$ (nM)
3	H	H2	F	2,300
EZE-gluc **1**	H	(S)-OH	F	530
EZE-gluc enantiomer **4**	H	(R)-OH	F	>100000
5	H	(R)-OH	F	3,900
6	H	=O	F	70,000
7	OH	(S)-OH	F	252
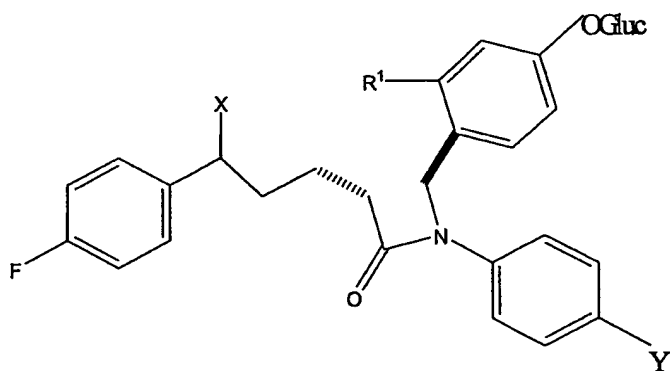
compounds **1**, **3**, **5**, **6** and **7**.
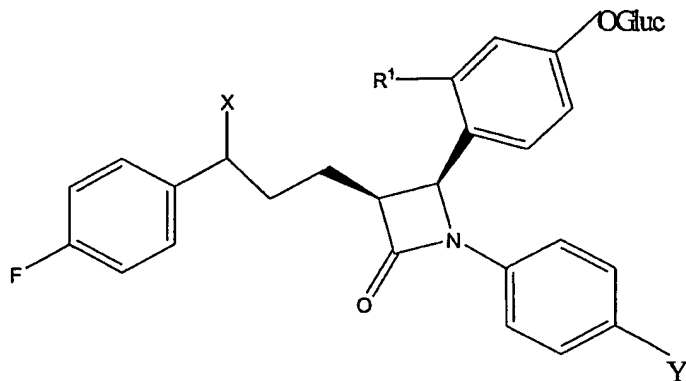
Backbone structure for compound **4**.

Example 31: Binding affinities of ezetimibe glucuronide and its analogs to recombinant NPC1L1

NPC1L1 was identified as a candidate target of ezetimibe from a

5   search of genetic databases for cholesterol binding motifs. Subsequently, *NPC1L1* deficient mice were found to have 80% reduction of cholesterol absorption, and did not respond to ezetimibe treatment, strongly suggesting that this protein is required for ezetimibe efficacy. To determine if NPC1L1 is the direct target of ezetimibe, binding affinities were compared for ezetimibe glucuronide and several analogs to

10   NPC1L1 transfected cells and rat brush border membrane vesicles.

Rat NPC1L1 transfected CHO cells (~500,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**. Compound **8** is an analog of compound **2** wherein the

15   hydroxyl group in the 3-hydroxylpropyl moiety of **2** is replaced with an oxo group.

Human NPC1L1 transfected CHO cells (~600,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) in buffer A for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**.

20   As shown in Figures 9 and 12, and Table 10, the affinities for the recombinant and native proteins are virtually identical, providing compelling evidence that NPC1L1 is the direct target of ezetimibe in mammalian tissues, and that other proteins are not required for binding.

Affinities of ezetimibe glucuronide and analogues thereof were also

25   determined for human recombinant NPC1L1. The results, shown in Figure 9, indicate that ezetimibe glucuronide (**1**) has an affinity for the human protein of 907 nM. The propargyl-sulfonamide analogue (**2**) is approximately 50-fold more potent, with a $K_d = 21$ nM, suggesting that this compound has the potential for enhanced potency of cholesterol absorption inhibition in man.

Table 10: Comparison of inhibition constants (Ki) for binding to native rat intestinal brush border membranes and membranes from rat NPC1L1 transfected cells.

Analog	Recombinant rat NPC1L1 Ki, nM	Rat BBMV Ki, nM
EZE-glucuronide **1**	790	600
2	12	15
3	2400	2300
6	84500	70000
5	5800	3900
8	556	818

5   **Example 32: Binding of ^{35}S-2 to membranes from wild type and NPC1L1 deficient mice.**

Final confirmation that NPC1L1 is the target of ezetimibe was provided by binding studies with ^{35}S-2 in intestinal brush border membranes from NPC1L1 deficient and control mice.

10   Brush border membranes vesicles were prepared from intestinal tissues of wild type and *NPC1L1* knockout (-/-) mice. 15, 30 and 60µg protein/ assay of brush border membranes vesicles were incubated with 4nM ^{35}S-2 in buffer A for 3 hours at 37°C in the presence and absence of 100µM EZE-glucuronide.

The results, shown in Figure 10, indicate that no detectable binding is 15   observed in membranes from NPC1L1 deficient mice, whereas age matched wild type control membranes have detectable binding. The binding affinity observed in this experiment in control mouse membranes (K_d=156 nM) was virtually identical to that observed in previous studies (Figure 11).

Example 33: Binding Analysis Using Brush Border Membrane Vesicles from
20   **Rat Mouse and Rhesus Monkey**

Binding studies were performed to compare the relative binding affinity of ezetimibe glucuronide to various brush border membrane vesicles.

^{3}H-ezetimibe glucuronide $\underline{1}$ was prepared as described in Example 27. The brush border membranes were prepared as described in Example 27.

Binding Assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 µl. In general, frozen membranes were diluted in buffer A or

5   buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.5 to 5 mg/ml (Buffer A: 26 mM NaHCO$_3$, 0.96 mM NaH$_2$PO$_4$, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4). Final concentrations of [^{3}H]ezetimibe glucuronide $\underline{1}$ were typically 25-50 nM , and were delivered as DMSO or CH$_3$CN solutions. Competing ligands were likewise added as DMSO solutions to

10   give a total 1-5 % organic solvent content. Nonspecific binding was defined by competition with 100-500 µM ezetimibe glucuronide. At least three components of buffer A, the bicarbonate and phosphate salts, and glucose, were later found to be inconsequential and were routinely omitted. Reactions were incubated until equilibrium was achieved (one hour for rat or three hours for rhesus membranes).

15   Bound ligand was recovered by single-tube vacuum filtration on Whatman GF/C glass fiber filters. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1 % sodium cholate, and 20 mM MES at pH 6.7) to the assay tube, pouring the mixture through the filter, and then

20   rinsing the tube and filter twice more with another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Ultima Gold MV liquid scintillation fluid from Packard. Where triplicate assays were performed, the standard error was typically <4%. As an example, a 100µl assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) [^{3}H]ezetimibe glucuronide gave 15,000 dpm specific and 3,000

25   dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

Data Analysis. After correction for nonspecific binding, saturation-binding data were fit by nonlinear regression (Sigma Plot) to the single-site expression $[B] = B_{max} \times [L]/([L] + K_D)$. Linear Scatchard plots are shown for

30   illustration. Data on K_i from competition experiments were analyzed by nonlinear regression to the expression $[B] = [B_O]/(1 + [I]/K_i^{obs})$, and where required were corrected for radioligand competition as $K_i = K_i^{obs}/(1 + [L^*]/K_D)$.

First-order rate constants (k_{obs} and k_{off}) were determined by nonlinear regression to the first order rate equation $A = A_o e^{-kt}$. Kinetic data for k_{on} were analyzed according to Weiland and Molinoff (32), using the equation $k_{on} = k_{obs}$ ($[LR]_e/([L] \, [LR]_{max})$), where $[L]$ is the concentration of ligand, $[LR]_e$ is the concentration of the complex at equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the apparent first-order rate constant.

Binding analysis. Binding studies using the [^{3}H]ezetimibe glucuronide a traditional rapid-filtration assay on glass fiber filters using enterocyte brush border membrane preparations from rat, mouse and rhesus monkey were performed (Table 11). Table 11 shows the binding affinities of [^{3}H]ezetimibe glucuronide to the membranes in the absence of detergents. The observed binding was specific, saturable, and consistent with a single molecular site. Scatchard analyses and the specific/nonspecific binding windows for rat and monkey are shown in Figure 12. The binding affinity is relatively weak in rat membranes ($K_D = 542$ nM) and even weaker in murine membranes ($K_D = 10,000$ nM). In contrast, binding affinity in rhesus monkey membranes is approximately 10-fold greater ($K_D = 41$ nM). The number of binding sites varied from $5 - 20$ pmol/mg membrane protein depending on species and preparation.

The rates for binding and dissociation of [^{3}H]ezetimibe glucuronide were determined and found to be slow relative to those typically observed for protein-ligand interactions. For example, the rate constants for association to rat and monkey brush border membranes are $k_{on} = 5.54$ and 3.90×10^3 M^{-1} s^{-1} (Figure 12). These are 100,000-fold smaller than those typically observed for a diffusion controlled encounter, 10^8 to 10^9 M^{-1} s^{-1}. Similarly, these complexes are unusually long-lived, dissociating with rate constants of $k_{off} = 2.4 \times 10^{-3}$ s^{-1} and 1.2×10^{-4} s^{-1} at 25C, equivalent to half-lives of 4.7 and 96 min for the rat and monkey complexes, respectively. In comparison, half lives are normally <1 sec for dissociation of common diffusion controlled, 100 nanopolar K_D ligands. These rate constants predict K_D values ($K_D = k_{off}/k_{on}$) of 440 and 32 nM, respectively, which agree well with those measured by equilibrium titration (Figure 12), and by saturation as described earlier. Such slow-forming, long-lived complexes suggest that conformational changes in the protein are rate limiting.

Table 11: Comparison of ezetimibe binding affinity and cross species efficacy

Species	K_D (nM)	ED_{50} (mg/Kg)
mouse	12,000	0.5000
rat	542	0.0300
monkey	41	0.0005

Table 11 also shows a correlation between *in vitro* and *in vivo* binding of [^{3}H]ezetimibe glucuronide in various enterocyte brush border membrane preparations from rat, mouse and rhesus monkey. The *in vivo* ED_{50} values are derived from cholesterol absorption and cholesterol feeding studies. The rank order of ezetimibe potency (ED_{50}) *in vivo* as follows: rhesus (0.0005mpk) > rat (0.03mpk) > mouse (0.5mpk) is the same as the order of *in vitro* binding affinity (IC_{50}) as follows: rhesus monkey (41nM) < rat (542nM) < mouse (12,000nM).

The binding affinities of **1** to brush border membranes correlate well across species with the sensitivity to ezetimibe inhibition of cholesterol uptake *in vivo* (mouse < rat < monkey) (Clader, J. W. The discovery of ezetimibe; A view from outside the receptor. *J Med. Chem.* 47, 1-9 (2004); Davis, H.R. Jr., Compton, D.S., Hoos, L. & Tetzloff, G. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 2032-2038 (2001); Burnett, D.A. Beta-lactam cholesterol absorption inhibitors. *Curr. Med. Chem.* 11, 1873-1887 (2004), consistent with the hypothesis that the assay is relevant to the target of ezetimibe *in vivo* (Table 11). As evidence that this interaction is very specific, the glucuronide of the enantiomer of ezetimibe was prepared and found to be completely inactive *in vitro* (K_i > 100 x K_D for ezetimibe glucuronide in all species), consistent with its lack of activity *in vivo* in a rat acute cholesterol absorption model (see Table 12 in which the enantiomer is analyzed).

Example 34: NPC1L1 as the target of ezetimibe in NPC1L1-expressing HEK293 cells.

This example demonstrates that ezetimibe binds specifically to NPC1L1-expressing HEK293 cells.

Transient expression of NPC1L1. Plasmid pCR3.1 expressing rat NPC1L1 (Genbank AY437867) or human NPC1L1 (Genbank AY437865) were

prepared using standard molecular biology protocols. HEK-293 cells (ATCC) were

seeded at 10 x 10^6 cells per T-225 flask (Corning) in DMEM containing 10% fetal

calf serum, 4.5 g/L D-glucose and L-glutamine, 18 hours prior to transfection. They

were transiently transfected with 25 μg of DNA using Fugene transfection reagent

5   (Roche Biochemical) at a ratio of 6:1 Fugene:DNA. Following transfection, the cells

were incubated at 37°C and 5% CO_2 for 48 hours, and then harvested using PBS

based cell dissociation buffer (Gibco), pelleted at 500 x g, snap frozen on dry ice, and

stored at -80°C.

           Membrane preparation from HEK-293 cells. Membranes were

10   prepared by resuspending the frozen cell pellets in ten volumes of 20 mM

HEPES/Tris buffer at pH 7.40 containing 8% sucrose, and sonicating the suspensions

with a probe sonicator on ice until most of the cells were lysed. To isolate the

membranes, the sonicates were centrifuged at 1600 x g for 10 min to remove cell

debris, and then the supernatants were centrifuged at 125,000 x g for 1 hour to recover

15   the membranes. These membranes were resuspended in 20 mM HEPES/Tris buffer at

pH 7.40 containing 160 mM NaCl and 5% glycerol, and stored at 10-20 mg/ml

protein at -80°C.

           Pursuing the recent evidence indicating that NPC1L1 is an important

component of the pathway inhibited by ezetimibe, recombinant rat and human

20   NPC1L1 were expressed in human embryonic kidney (HEK) 293 cells (Figure 13,

Panel 1). Cell lysates from HEK-293 cells expressing NPC1L1 (Lanes 1 and 3 of

Panel 1 Figure 13) and wild-type cells (Lanes 2 and 4 of Panel 1 Figure 13) were

analyzed by gel electrophoresis and Western blot with an anti-NPC1L1 antibody

A1801. An excess of NPC1L1-specific peptide was included to assess specificity of

25   the antibody for NPC1L1 (Lane 3 and 4 of Panel 1 Figure 13). Preliminary binding

studies using 1 revealed specific binding to membrane preparations from cells

expressing NPC1L1, and no specific binding to membranes from mock transfected

cells (not shown).

           Binding to NPC1L1 expressing cells was also observed with a

30   BODIPY-labeled fluorescent ezetimibe glucuronide analog (SCH354909) (Figure 13,

Panel 2A). Panel 2 of Figure 13 shows confocal microscope images of a fluorescent

ezetimibe glucuronide analog (SCH354909) bound to the surface of NPC1L1-293

cells (Panel 2A), nonspecific binding of SCH354909 to NPC1L1-293 cells in the

presence of 100 μM unlabeled ezetimibe glucuronide (Panel 2B), binding of
SCH354909 to wild type HEK 293 cells (Panel 2C), and nonspecific binding of
SCH354909 to wild type HEK 293 cells in the presence of 100 μM unlabeled
ezetimibe glucuronide (Panel 2D). In each case, plated cells were incubated in culture

5   media with 500 nM SCH354909 for 4 hours at 37°C. Cells were subsequently
washed with PBS and fluorescence was detected using confocal microscopy.

Binding of SCH345909 was clearly evident at the cell surface
membrane of the NPC1L1-expressing cells and was completely abolished in the
presence of excess unlabeled ezetimibe glucuronide (Figure 13, Panel 2C). No

10   binding was observed in wild type HEK 293 cells (Figure 13, Panels 2B and 2D).
These results demonstrated that ezetimibe glucuronide binds specifically to NPC1L1.

Example 35: NPC1L1 as the in vivo target of ezetimibe.

To obtain evidence that NPC1L1 is the direct binding target of
ezetimibe *in vivo*, binding affinities of 1 and several key analogs were determined for

15   recombinant rat and human NPC1L1 expressed in HEK-293 cell membranes and
compared to those for native rat and rhesus intestinal enterocyte brush border
membranes. A series of ezetimibe analogs was selected with subtle structural
diversity, but with no binding affinities to native brush border membranes that
covered a range of 1000-fold.

20   Table 12 shows a comparison of binding affinities (K_i values) for
recombinant NPC1L1-293 cell membranes and native brush border membranes
Selected analogs of ezetimibe glucuronide are compared against recombinant rat and
human NPC1L1 membranes prepared from transiently transfected HEK-293 cells
compared to native rat and rhesus brush border membranes. The binding assays were

25   conducted in a final volume of 20 μl in the presence of 0.03% sodium taurocholate
and 0.05% digitonin until equilibrium was achieved. 1.25 mg protein/ml and 100 nM
1 were used for native rat, recombinant rat, and recombinant human experiments, and
1.25 mg protein/ml and 20 nM 1 were used for native rhesus monkey experiments.
Observed total and nonspecific binding, respectively, in the absence of inhibition

30   were native rat: 7,700 & 1,100, recombinant rat: 33,000 & 1,100, native rhesus
monkey: 7,300 & 367, and recombinant human: 19,200 & 1,000 dpm. Analog
structures are defined in Table 12. Compound 4 has the stereochemical configuration
3S, 4R, and is the glucuronide of the enantiomer of ezetimibe. These determinations

were conducted in buffer containing 0.03% taurocholate and 0.05% digitonin, levels

below the critical micelle concentrations of these detergents. These conditions

enhanced apparent binding by as much as 20-fold for the recombinant preparations

(principally a B_{max} effect), and greatly facilitated a quantitative comparison of K_i

5   values for $\underline{1}$ and its analogs.

As shown in Table 12, the K_i values for recombinant rat NPC1L1 and

native rat brush border membranes are virtually identical, strongly suggesting that

NPC1L1 is the molecular target of ezetimibe *in vivo*. In the case of membranes from

cells expressing recombinant human NPC1L1, the binding affinities also parallel

10   those observed in rat membranes, whereas binding affinities for native rhesus brush

border membranes are uniformly ~10-fold more potent. This result is consistent with

the finding that ezetimibe is an order of magnitude more potent in monkey than in

human or rat (Clader, J. W. The discovery of ezetimibe; A view from outside the

receptor. *J Med. Chem.* 47, 1-9 (2004); Jeu, L. & Cheng, J.W. Pharmacology and

15   therapeutics of ezetimibe (SCH 58235), a cholesterol-absorption inhibitor. *Clin. Ther.*

25, 2352-2387 (2003)).

TABLE 12

5
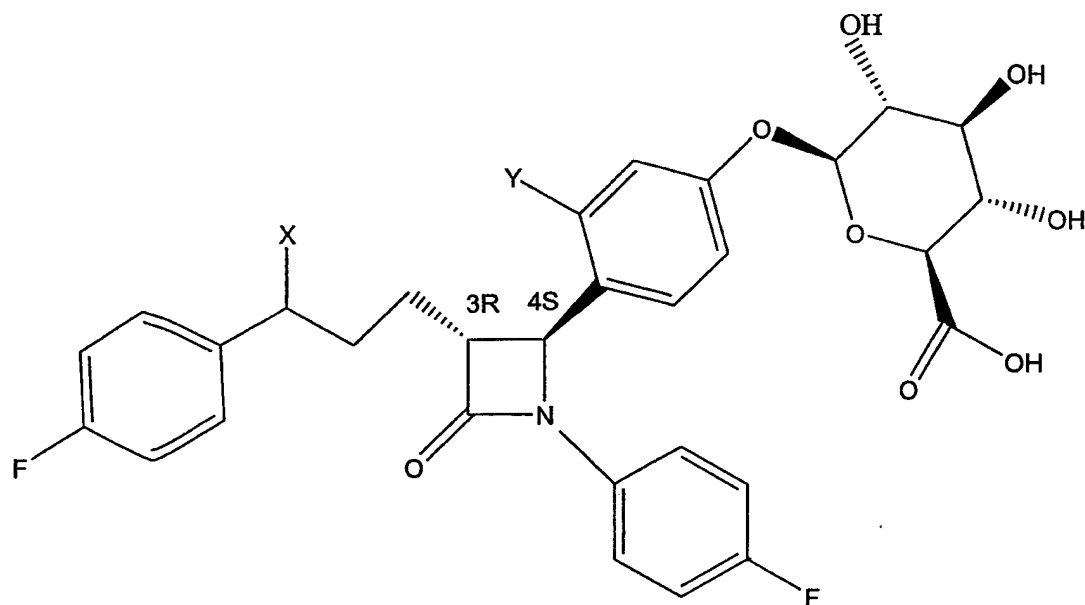
Analog	X	Y	Rat	Rat	Human	Monkey
			BBM Ki (nM)	NPC1L1 Ki (nM)	NPC1L1 Ki (nM)	BBM Ki (nM)
EZE-gluc **1**	OH (S)	H	390	210	220	15
4*	OH (R)	H	130,000	74,000	130,000	22,000
3	H	H	1,600	820	1,000	150
6	=O	H	33,000	23,000	14,000	3,300
5	OH (R)	H	2,800	1,700	1,300	120
7	OH (S)	OH	280	360	210	60

* glucuronide of the enantiomer of ezetimibe has stereochemical configuration 3*S*, 4*R*

Conclusive evidence that NPC1L1 is the target of ezetimibe was provided by studies with tissues from NPC1L1 deficient mice. Enterocyte brush border membranes prepared from NPC1L1 deficient mice showed no detectable specific binding affinity for 1, whereas membranes from age-matched wild-type mice

5   showed a high level of specific binding with a $K_D = 12 \ \mu M$ (Figure 14).

For Figure 14A, enterocyte brush border membranes were prepared from NPC1L1 deficient male mice and same sex wild-type littermates, and tested for binding of 1. Conditions for binding were 5 mg/ml protein and 500 nM 1 in a volume of 20 μl and in the presence of 0.03% sodium taurocholate and 0.05% digitonin.

10   Membranes from wild type mice are on the left and from NPC1L1 deficient mice on the right. The bar graphs indicate total binding (left bar), nonspecific binding in the presence of 500 μM cold ezetimibe glucuronide (middle bar), and specific (right bar) binding, respectively for each of wild type and NPC1L1 deficient mice, and error bars represent triplicate measurements. The graphs show that although specific binding is

15   readily detectable in wild-type mice, it is absent in NPC1L1-deficient mice.

Figure 14B shows a plot demonstrating competition of unlabeled ezetimibe glucuronide against 1. Membranes from wild-type mice (upper curve) gave $K_i = 12,000$ nM, while specific binding was virtually undetectable in membranes from the knockout animals (lower curve). Conditions were those described in Figure 14A.

20   The present studies involve a quantitative comparison of binding between recombinant proteins and brush border membranes. SR-BI (scavenger receptor type B1) was previously identified as a potential target using an expression cloning strategy employing ezetimibe binding to candidate proteins; this hypothesis was readily dismissed when neither cholesterol absorption nor ezetimibe activity were

25   affected in SR-BI deficient mice (Altmann, S. W. *et al.* The identification of intestinal scavenger receptor class B, type 1 (SR-B1) by expression cloning and its role in cholesterol absorption. *Biochem. Biophs. Acta* 1580, 77-93 (2002)). The results show that ezetimibe binds to native intestinal membranes and cells expressing recombinant NPC1L1 with comparable affinity, and does not bind to membranes from NPC1L1

30   deficient mice, indicating a specific binding interaction between NPC1L1 and ezetimibe. Together with the previously published findings that mice deficient in NPC1L1 are defective in intestinal cholesterol uptake, and are no longer responsive to ezetimibe (Altmann, S. W. *et al.* Niemann-Pick C1 Like 1 protein is critical for

intestinal cholesterol absorption. *Science* 303, 1201-1204 (2004)), these data
definitively establish NPC1L1 as the direct target of ezetimibe.

Example 36: Effect of detergents on [^{3}H]ezetimibe glucuronide binding.

5 A practical aspect of work with the recombinant protein was that the
number of binding sites in transfected NPC1L1-293 cell membranes initially appeared
quite low. The influence of a combination of 0.03% taurocholate and 0.05% digitonin
on specific binding to these and native enterocyte brush border membrane
preparations is dramatic as illustrated in Figure 15.

Equal amounts (25 µg protein) of rat brush border membranes,
10 membranes from HEK-293 cells transiently expressing recombinant rat and human
NPC1L1, were incubated with 25 nM 1 in a final volume of 20 µl until equilibrium
was achieved. The incubation conditions were buffer A with and without sodium
taurocholate and digitonin to a final concentration of 0.03% and 0.05%, respectively.
On the x-axis, "C" denotes controls in the absence of detergent, and "+det" the
15 response in the presence of both detergents. The results are shown in 3 bar groupings;
Total binding (left bar in each 3 bar group), nonspecific binding in the presence of
100 µM unlabeled ezetimibe glucuronide (middle bar in each 3 bar group), and
specific binding (right bar in each 3 bar group) are shown.

Example 37: Binding affinities of ezetimibe glucuronide and various analogues
20 **NPC1L1 in rat and rhesus monkey membranes.**

As determined from binding assay results using ^{3}H-ezetimibe
glucuronide with rat brush border membrane, representative tested compounds of
Formula II were determined to have IC50's of about 13,000nM or lower, and
particularly certain tested compounds had IC50's of about 1900nM or lower, more
25 particularly certain tested compounds had IC50's of about 1000nM or lower, and
most particularly certain tested compounds had IC50's of less than 100nM. As
determined from binding assay results using ^{3}H-ezetimibe glucuronide with rhesus
brush border membrane, representative tested compounds of Formula II were
determined to have IC50's of about 4200nM or lower, and particularly certain tested
30 compounds had IC50's of about 165nM or lower, more particularly certain tested
compounds had IC50's of less than 100nM, and most particularly certain tested
compounds had IC50's of less than 50nM.

The designations below are used in the Examples that follow for certain repetitively used intermediates:
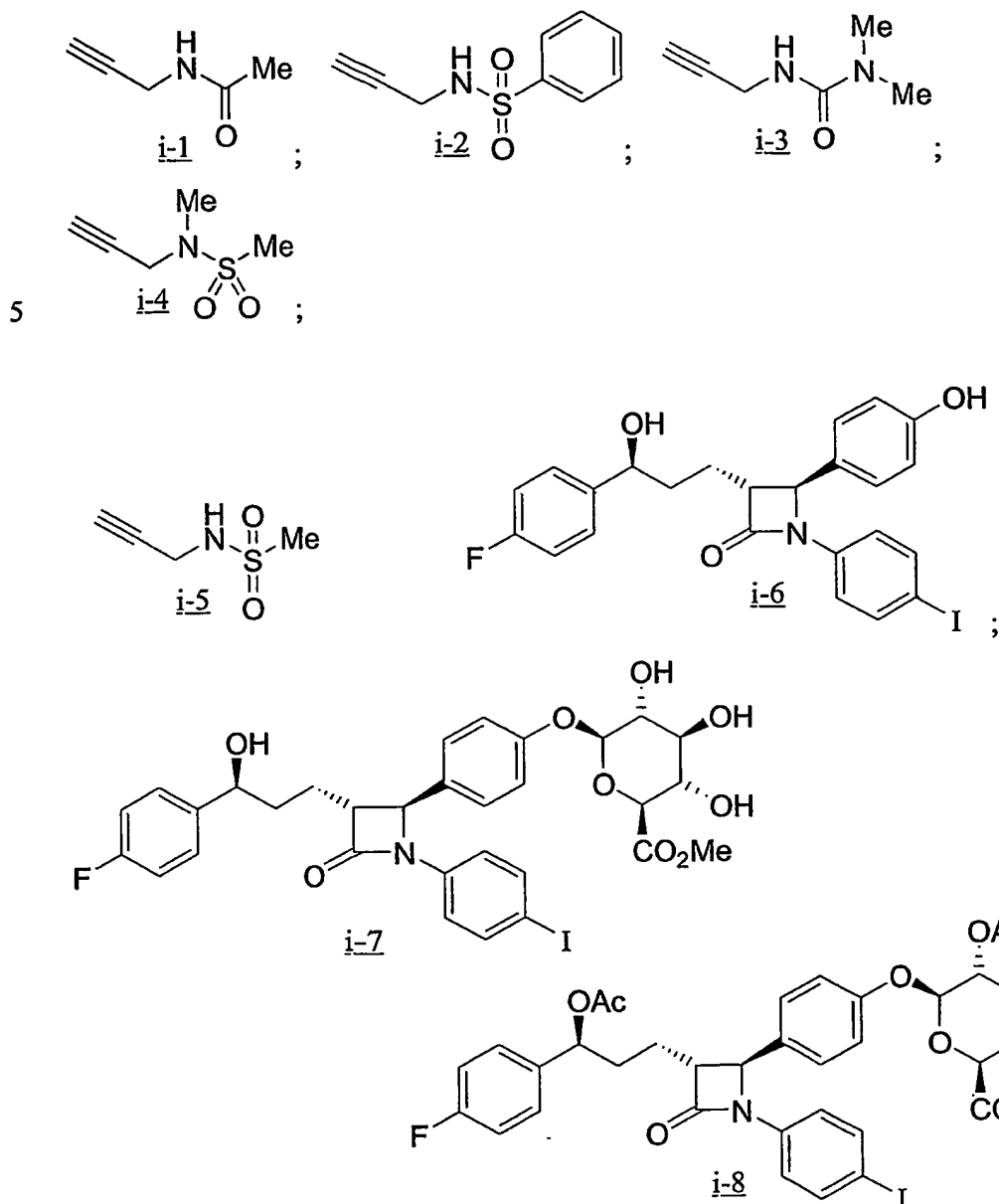
The compounds (3R,4S)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (i-6) and 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (i-7) were prepared according to Burnett, D. S.; Caplen, M. A.; Domalski, M. S.; Browne, M. E.; Davis, H. R. Jr.; Clader, J. W. *Bioorg. Med. Chem. Lett.* (2002), *12*, 311. Compound i-8 is the hydroxy-protected analog of i-7, where the protecting group is acyl.

The following definitions are also used in the Examples that follow:

W = [structure: a chemical structure showing a 4-fluorophenyl group with OAc substituent, an azetidinone ring, and a phenyl-linked pyranose ring bearing OAc, OAc, OAc and CO$_2$Me groups]

W^1 = [structure: a chemical structure showing a 4-fluorophenyl group with OH substituent, an azetidinone ring, and a phenyl-linked pyranose ring bearing OH, OH, OH and CO$_2$Me groups]

W^2 = [structure: a chemical structure showing a 4-fluorophenyl group with OH substituent, an azetidinone ring, and a phenyl-linked pyranose ring bearing OH, OH, OH and CO$_2$H groups]

EXAMPLE 38

5   Preparation of *N*-prop-2-yn-1-ylacetamide (i-1)

Acetyl chloride (0.52 mL, 7.3 mmol) was added to a stirred solution of propargylamine (0.5 mL, 7.3 mmol) and dimethylaminopyridine (18 mg, 0.14 mmol) in pyridine (2.5 mL) at 0°C, and the resulting mixture was allowed to warm to ambient temperature. After approximately 15 h, the reaction mixture was diluted with

10   ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na$_2$SO$_4$), filtered and concentrated *in vacuo* to afford the title compound (i-1), which was used without further purification.

EXAMPLE 39

Preparation of *N*-prop-2-yn-1-ylbenzenesulfonamide (i-2)

15   Benzene sulfonyl chloride (1.16 mL, 9.1 mmol) was added to stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting solution was aged

at ambient temperature for approximately 15 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na₂SO₄), filtered and concentrated *in vacuo* to furnish the title compound (i-2), which was used without further purification.

5   EXAMPLE 40

Preparation of *N,N*-Dimethyl-*N'*-prop-2-yn-1-ylurea (i-3)

                Dimethyl carbamylchloride (0.84 mL, 9.1 mmol) was added to a stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting
10   suspension was stirred at ambient temperature for approximately 15 h. The reaction . mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na₂SO₄), filtered and concentrated *in vacuo* to afford the title compound (i-3), which was used without further purification.

                                     EXAMPLE 41

15   Preparation of *N*-Methyl-*N*-prop-2-yn-1-ylmethanesulfonamide (i-4)

                Methansulfonylchloride (1.12 mL, 14.5 mmol) was added to a stirred solution of *N*-methylpropargylamine (1.22 mL, 14.5 mmol) and dimethylaminopyridine (35 mg, 0.30 mmol) in pyridine (10 mL) at room temperature. After aging for approximately 15 h, the reaction mixture was poured into ethyl acetate
20   and washed successively with 1N HCl and brine. The organic phase was dried (Na₂SO₄), filtered and concentrated *in vacuo*, to afford the title compound (i-4), which was used without further purification.

                                     EXAMPLE 42

Preparation of *N*-prop-2-yn-1-ylmethanesulfonamide (i-5)
25   Methansulfonylchloride (1.40 mL, 18.1 mmol) was added dropwise to a stirred solution of propargylamine (1.00 g, 18.1 mmol) and dimethylaminopyridine (44.0 mg, 0.36 mmol) in pyridine (10 mL) at 0 °C. After aging for approximately 15 h, the reaction mixture was poured into 1N HCl and extracted twice with ethyl acetate. The combined organic extracts were washed with saturated aqueous sodium
30   bicarbonate, brine, dried (MgSO₄), filtered and concentrated *in vacuo*, to afford the

title compound i-5. Crude i-5 crystallized on standing and was used without further purification.

EXAMPLE 43

Preparation of *N*-(3-{4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-2-(4-hydroxyphenyl)-4-oxoazetidin-1-yl]phenyl}prop-2-yn-1-yl)methanesulfonamide (Compound 6a)
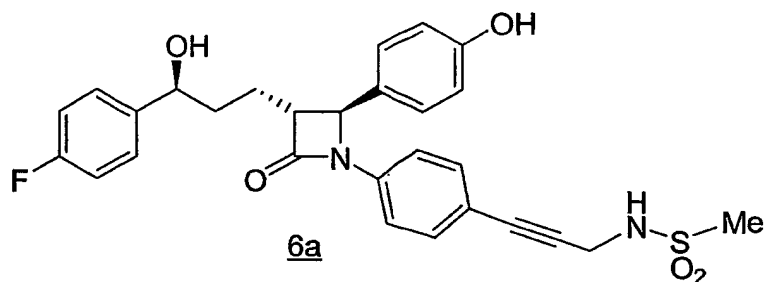
Triethylamine (7 equivalents) is added to a solution of (3*R*,4*S*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (i-6) (1.00 equivalent), *N*-prop-2-yn-1-ylmethanesulfonamide (i-5) (1.50 equivalents), tetrakistriphenylphosphine palladium(0) (0.15 equivalents) and copper(I) iodide (0.30 equivalents) in DMF (0.1 M concentration with respect to final product) under a nitrogen atmosphere and the resulting solution aged at room temperature. After completion of reaction, the volatiles are evaporated in vacuo and the crude residue can be purified by flash chromatography on silica gel to afford the title compound.

EXAMPLE 44

Step A: Preparation of 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-
             hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-
             yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-
5   glucopyranosiduronate (Compound 7a)

7a
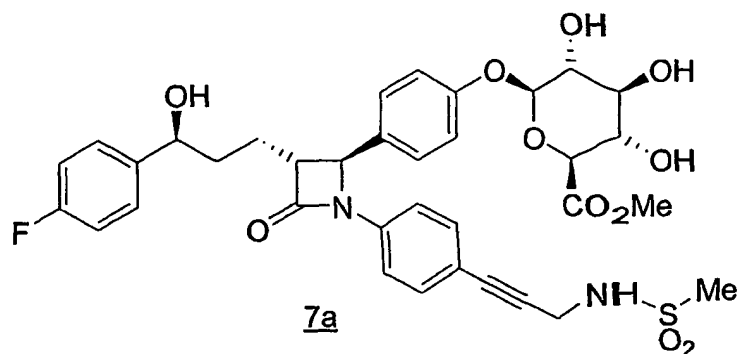
Triethylamine (0.07 mL, 0.502 mmol) was added to a stirred solution
of 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-
oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (i-7) (0.050 g, 0.071
10   mmol), N-prop-2-yn-1-ylmethanesulfonamide (i-5) (0.014 g, 0.105 mmol),
tetrakistriphenylphosphine palladium(0) (0.012 g, 0.010 mmol) and copper iodide
(0.005 g, 0.026 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the
resulting solution aged at room temperature for 18 h. The volatiles were evaporated
in vacuo and the crude residue purified by flash chromatography on silica gel
15   (gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title
compound; m/z (ES) 713 (MH+), 505.

Step B: Preparation of 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-
hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-
yl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid
(Compound 7b, also referred to herein as compound 2)

7b
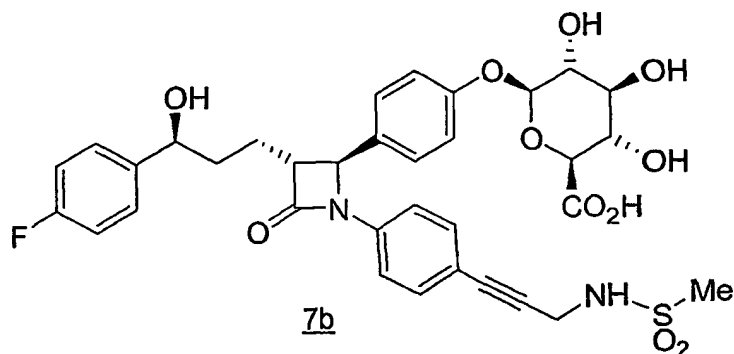
A solution of compound 7a in methanol/water/triethylamine (1:7:2;
1 mL) was stirred at room temperature for approximately 1.5 h. The volatiles were
evaporated *in vacuo* and the crude residue purified by preparative reversed phase high
performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution;
10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound
(7b); m/z (ES) 699 (MH$^+$), 505; HRMS (ES) m/z calcd for $C_{34}H_{36}FN_2O_{11}S$ (MH$^+$)
699.2024, found 699.2016.

EXAMPLE 45 (COMPOUNDS 6B TO 6G AND 7C TO 7N)

The following compounds of Formula IIa have been prepared (as
indicated by MS data provided) or can be prepared using the general synthetic
procedures described in Example 43 (shown in Table 13) or Example 44 (shown in
Table 14).

IIa
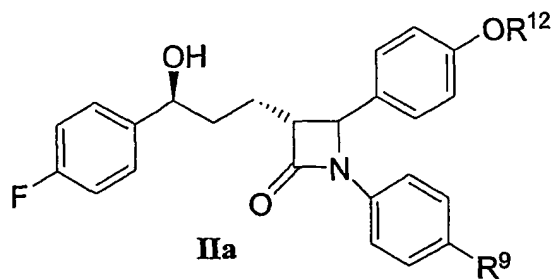
TABLE 13

Compound	R^{12}	R^9
6b)	H	
6c)	H	
6d)	H	
6e)	H	
6f)	H	
6g)	H	

TABLE 14
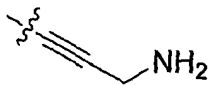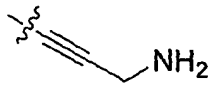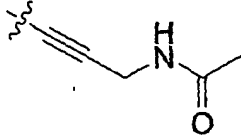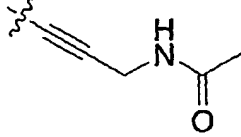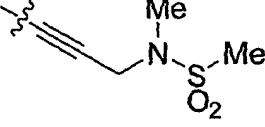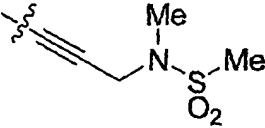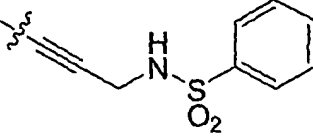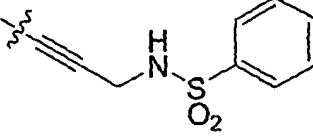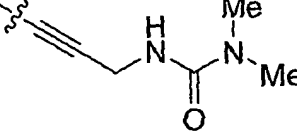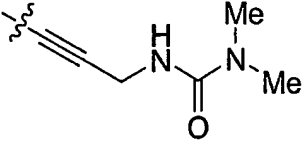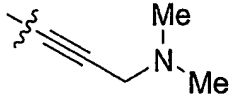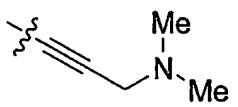
Compound	R12	R9	m/z (ES)	HRMS m/z (ES)
7c)	methyl ester glucuronide	—C≡C—CH$_2$NH$_2$ (propargyl, NH$_2$)	658 (MNa$^+$)	
7d)	glucuronide	—C≡C—CH$_2$NH$_2$	621 (MH$^+$)	(MH$^+$) Calcd 621.2249 Found 621.2223
7e)	methyl ester glucuronide	—C≡C—CH$_2$NH—C(=O)CH$_3$	677 (MH$^+$)	
7f)	glucuronide	—C≡C—CH$_2$NH—C(=O)CH$_3$	663 (MH$^+$)	(MH$^+$) Calcd 663.2354 Found 663.2331
7g)	methyl ester glucuronide	—C≡C—CH$_2$—N(Me)—SO$_2$—Me	749 (MNa$^+$)	
7h)	glucuronide	—C≡C—CH$_2$—N(Me)—SO$_2$—Me	735 (MNa$^+$)	(MH$^+$) Calcd 713.2180 Found 713.2170
7i)	methyl ester glucuronide	—C≡C—CH$_2$—NH—SO$_2$—Ph	797 (MNa$^+$)	
7j)	glucuronide	—C≡C—CH$_2$—NH—SO$_2$—Ph	783 (MNa$^+$)	(MH$^+$) Calcd 761.2180 Found 761.2193
7k)	methyl ester glucuronide	—C≡C—CH$_2$—NH—C(=O)—N(Me)—Me	706 (MH$^+$)	

TABLE 14

Compound	R12	R9	*m/z* (ES)	HRMS *m/z* (ES)
7l)	glucuronide		692 (MH⁺)	(MH⁺) Calcd 692.2620 Found 692.2618
7m)	methyl ester glucuronide		663 (MH⁺)	
7n)	glucuronide		649 (MH⁺)	(MH⁺) Calcd 649.2562 Found 649.2532

EXAMPLE 46

Step A:　　　Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-
hydroxypropyl]-4-oxo-1-{4-[(trimethylsilyl)ethynyl]phenyl}azetidin-
2-yl)phenyl methyl β-D-glucopyranosiduronate (8a)
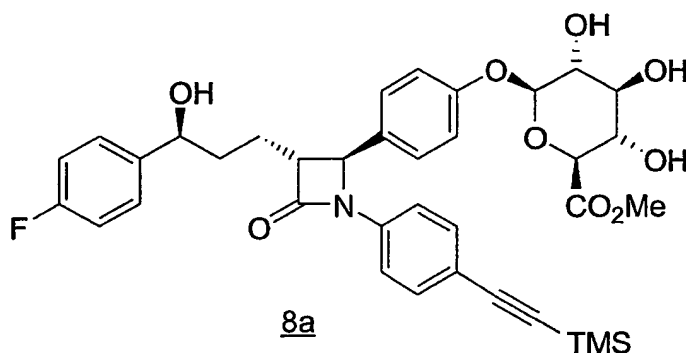
Triethylamine (69.0 μL, 0.495 mmol) was added to a stirred solution
of i-7 (50.0 mg, 0.071 mmol), trimethylsilyacetylene (12.0 μL, 0.085 mmol),
tetrakistriphenylphosphine palladium(0) (13.0 mg, 0.011 mmol) and copper iodide
(5.10 mg, 0.028 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the
resulting solution aged at room temperature for 18 h. The volatiles were evaporated
in vacuo and the crude residue purified by flash chromatography on silica gel

(gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title

compound (8a); *m/z* (ES) 660 (M-OH)$^+$, 470.

Step B: Preparation of 4-{(2*S*,3*R*)-1-(4-ethynylphenyl)-3-[(3*S*)-3-(4-
            fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β-D-
            glucopyranosiduronic acid (8b)

8b
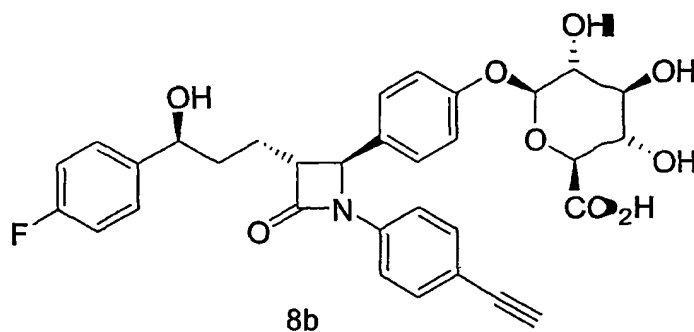
A solution of **8a** in methanol/water/triethylamine

(0.25 mL:1.10 mL:0.40 mL) was stirred at room temperature for approximately 6 h.

The volatiles were evaporated *in vacuo* and the crude residue purified by preparative

reversed phase high performance liquid chromatography on YMC Pack Pro C18

phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to

give the title compound (8b); *m/z* (ES) 574 (M-OH)$^+$, 398; HRMS (ES) *m/z* calc'd for

$C_{32}H_{31}FNO_9$ (MH$^+$) 592.1983, found 592.1985.

EXAMPLE 47

Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-
            hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-
            oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (9a)

9a
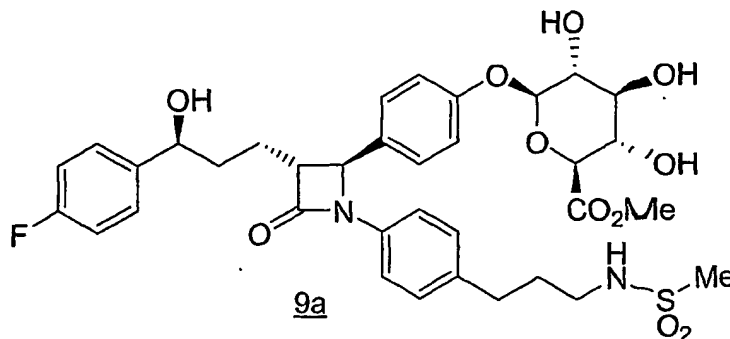
A mixture of 7a (40.0 mg, 0.056 mmol) and palladium (~8 mg of 10 wt. % (dry basis) on activated carbon) in methanol (2 mL) was hydrogenated at atmospheric pressure for approximately 1 h. The reaction mixture was filtered through a short plug of celite, eluting copiously with methanol, and the filtrate evaporated *in vacuo* to afford the title compound (9a); *m/z* (ES) 509 (M-sugar-OH)$^+$.

Step B: Preparation of 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (9b)

9b
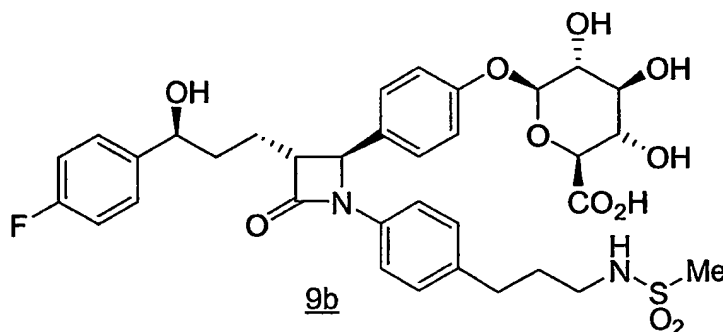
A solution of 9a in methanol/water/triethylamine (1:7:2, 1 mL) was stirred at room temperature for approximately 1 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (9b); *m/z* (ES) 735 (M+Na)$^+$, 685 (M-OH)$^+$, 509 (M-sugar-OH)$^+$; HRMS (ES) *m/z* calc'd for C$_{34}$H$_{39}$FN$_2$O$_{11}$S (MH$^+$) 703.2337, found 703.2337.

EXAMPLE 48

Step A: Preparation of 4-{(2S,3R)-3-[(3S)-3-acetoxy)-3-(4-fluorophenyl)propyl]-1-[4-(3-{[tert-butyl(dimethylsilyl]oxy}prop-1-yn-1-yl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (10a)

10a
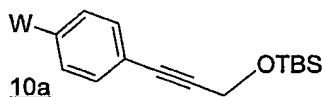
Triethylamine (170 µL, 1.25 mmol) was added to a solution of i-8 (156 mg, 0.178 mmol), *tert*-butyldimethyl(2-propynyloxy)silane (43.0 µL, 0.214 mmol),

dichlorobistriphenylphosphine palladium(II) (12.0 mg, 0.018 mmol) and copper

iodide (7.00 mg, 0.036 mmol) in DMF (1.3 mL) under a nitrogen atmosphere and the

resulting solution aged at room temperature for approximately 20 h. The reaction

mixture was poured into saturated aqueous sodium bicarbonate and extracted twice

5   with diethyl ether. The combined organic extracts were washed with water, brine,

dried (MgSO₄), filtered and the filtrate concentrated *in vacuo*. Purification of the

crude residue by flash chromatography on silica gel (gradient elution; 15-40% ethyl

acetate/hexanes as eluent) afforded the title compound 10a.

Step B: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-

10   (4-fluorophenyl)propyl]-1-[4-(3-hydroxyprop-1-yn-1-

                        yl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl

                        2,3,4-tri-*O*-acetyl-β–D-glucopyranosiduronate (10b)

10b
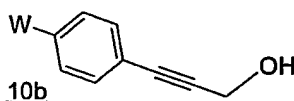
Tetrabutylammonium fluoride hydrate (39.0 mg, 0.148 mmol) was

15   added to 10a (136 mg, 0.148 mmol) in tetrahydrofuran (1.5mL), and the resulting

solution aged at room temperature for 1 h. The reaction mixture was poured into

saturated aqueous ammonium chloride and extracted twice with ether. The combined

organic extracts were washed with saturated sodium bicarbonate, brine, dried

(MgSO₄), filtered and concentrated *in vacuo*. Purification of the crude residue by

20   flash chromatography on silica gel (50% ethyl acetate/hexanes) afforded the title

compound 10b.

Step C: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-

                        3-(4-fluorophenyl)propyl]-4-oxo-1-[4-(3-oxoprop-

                        1-yn-1-yl)phenyl]azetidin-2-yl}phenyl methyl

25   2,3,4-tri-*O*-acetyl-β–D-glucopyranosiduronate (10c)

10c
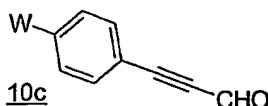
Dess-Martin periodinane (33.0 mg, 0.077mmol) was added to a

solution of 10b (62.0 mg, 0.077 mmol) and pyridine (31.0 μL, 0.386 mmol) in

dichloromethane (1 mL) at room temperature. After 30 min, the reaction mixture was

poured into saturated aqueous sodium bicarbonate, and extracted twice with ethyl

acetate. The combined organic extracts were washed with water, brine, dried

(MgSO₄), filtered and concentrated *in vacuo*. Purification of the crude residue by

flash chromatography on silica gel (gradient elution; 20-40% ethyl acetate/hexanes)

5   afforded the title compound <u>10c</u>.

Step D: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-

fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-

yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d)
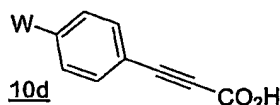
10   An aqueous solution (0.1 mL) of sodium dihydrogenphosphate (9.00

mg, 0.065 mmol) and sodium chlorite (5.00 mg, 0.055 mmol) was added to a solution

of <u>10c</u> (37.0 mg, 0.046 mmol) in *tert*-butyl alcohol (0.4 mL), dioxane (0.2 mL) and

isobutylene (~0.1 mL) at room temperature. After 1.5 h, the reaction mixture was

concentrated *in vacuo* and the crude residue triturated repeatedly with ethyl acetate.

15   The organic washings were dried (Na₂SO₄), filtered and concentrated *in vacuo* to

afford the title compound <u>10d</u>.

Step E: Preparation of 4-{(2*S*,3*R*)-1-[4-(carboxyethynyl)phenyl]-3-[(3*S*)-

3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl

β–D-glucopyranosiduronic acid (10e)
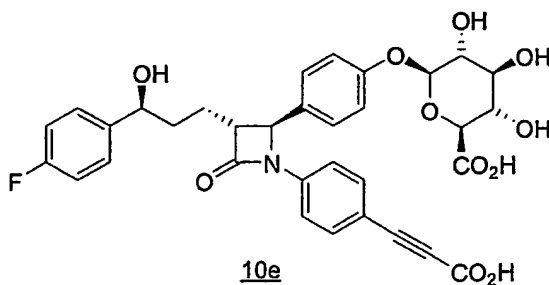
20   A solution of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-

fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl}phenyl

methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (<u>10d</u>) and sodium cyanide

(~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 22 h, the reaction

25   mixture was concentrated under reduced pressure and dissolved in

methanol/water/triethylamine (1:7:2, 1 mL). After stirring at room temperature for

approximately 1 h, the volatiles were evaporated *in vacuo* and the crude residue

purified by preparative reversed phase high performance liquid chromatography on

YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent,

0.1% TFA modifier) to give the title compound (10e), *m/z* (ES) 442.0 (M-sugar-

5   OH)$^+$, 618.0 (M-OH)$^+$; HRMS (ES) *m/z* calcd. for $C_{33}H_{31}FNO_{11}$ (MH$^+$) 636.1881,

found 636.1889

EXAMPLE 49

Step A: Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-

fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-

10   :   1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl

2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (11a)
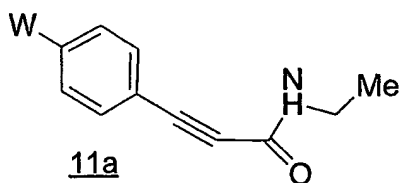
A 1M solution of ethylamine hydrochloride and diisopropylethylamine

in DMF (40.0 μL, 0.40 mmol) was added to 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-

15   fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl}phenyl

methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d) (27.0 mg, 0.033 mmol),

1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (19.0 mg, 0.099

mmol) and 1-hydroxybenzotriazole (8.00 mg, 0.059 mmol) in DMF (0.25 mL). After

4.5 h, the reaction mixture was poured into ethyl acetate and washed successively

20   with water and brine. The organic layer was dried, filtered and concentrated under

reduced pressure. Purification of the crude residue by flash chromatography on silica

gel (gradient elution; 50-60% ethyl acetate/hexanes) afforded the title compound 11a.

Step B: <u>Preparation of 4-{(2S,3R)-1-{4-[3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β-D-glucopyranosiduronic acid (11b)</u>
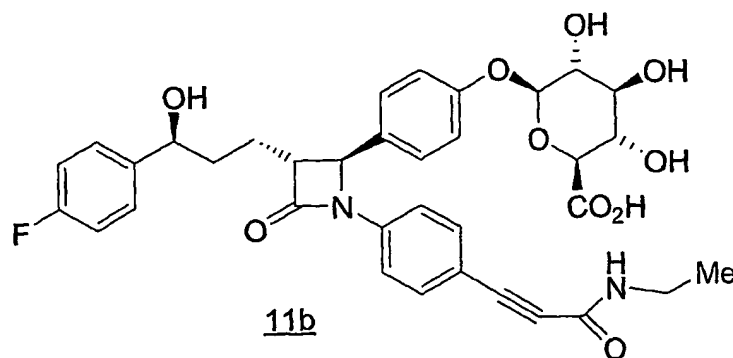
<u>11b</u>

A solution of 4-((2S,3R)-3-[(3S)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate (<u>11a</u>) (22.0 mg, 0.026 mmol) and sodium cyanide (~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 18 h, the reaction mixture was concentrated under reduced pressure and dissolved in methanol/water/triethylamine (1:3:1, 2.5 mL). After stirring at room temperature for approximately 1 h, the volatiles were evaporated *in vacuo*, and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (<u>11b</u>) *m/z* (ES) 663.0 (M+H)$^+$; HRMS (ES) *m/z* calcd. for $C_{35}H_{36}FN_2O_{10}$ (MH$^+$) 663.2354, found 663.2341.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, Genbank Accession Numbers and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

CLAIMS

1. A method for identifying a ligand of NPC1L1 comprising:

contacting human NPC1L1 with a detectably labeled substituted 2-azetidinone

glucuronide and a candidate compound; and

determining whether said candidate compound binds to human NPC1L1;

wherein binding of said candidate compound to human NPC1L1 modulates

binding of said detectably labeled substituted 2-azetidinone glucuronide to human

NPC1L1, wherein the detectably labeled substituted 2-azetidinone glucuronide has a

binding affinity K_D value for human NPC1L1 that is 200nM or lower, and wherein

said modulation indicates that the candidate compound is a ligand that binds to human

NPC1L1.

2. The method of claim 1, wherein the K_D value is 100nM or lower.

3. The method of claim 1, wherein the K_D value is 50nM or lower.

4. The method of claim 1, wherein the K_D value is 20nM or lower.

5. The method of claim 1, wherein the K_D value is 10nM or lower.

6. The method of claim 1, wherein the substituted 2-azetidinone-glucuronide

is selected from the group consisting of a compound of Formula I and a compound of

Formula II.

7. The method of claim 6, wherein the substituted 2-azetidinone-glucuronide

comprises a detectable label from the group consisting of ^{35}S and ^{125}I.

8. The method of claim 7, wherein the detectable label is ^{35}S.

9. The method of claim 6, wherein the substituted 2-azetidinone-glucuronide

is a compound of Formula II, wherein R^9 comprises an $-SO_2-$ group.

10. The method of claim 9, wherein the substituted 2-azetidinone-glucuronide

of Formula II is labeled with ^{35}S.

11. A method for identifying a ligand of NPC1L1 comprising:

contacting human NPC1L1 with a detectably labeled substituted 2-azetidinone glucuronide of Formula II and a candidate compound; and

determining whether said candidate compound binds to human NPC1L1;

wherein binding of said candidate compound to human NPC1L1 modulates

binding of said detectably labeled substituted 2-azetidinone glucuronide of Formula II

to human NPC1L1, and wherein said modulation indicates that the candidate

compound is a ligand that binds to human NPC1L1.

12. The method of claim 11, wherein R^9 of the detectably labeled substituted

2-azetidinone glucuronide of Formula II comprises an -SO_2- group.

13. The method of claim 11, wherein the detectably labeled substituted 2-azetidinone glucuronide of Formula II is labeled with [35]S.

14. The method of claim 11, wherein the detectably labeled substituted 2-azetidinone glucuronide of Formula II has a binding affinity K_D value for human NPC1L1 that is 200nM or lower.

15. The method of claim 14, wherein the K_D value is 100nM or lower.

16. The method of claim 14, wherein the K_D value is 50nM or lower.

17. The method of claim 14, wherein the K_D value is 20nM or lower.

18. The method of claim 14, wherein the K_D value is 10nM or lower.

19. The method of claim 1 wherein the detectably labeled substituted 2-azetidinone glucuronide is labeled with [35]S.

20. The method of claim 1 wherein the detectably labeled substituted 2-azetidinone glucuronide is ^{35}S-labeled compound 2.
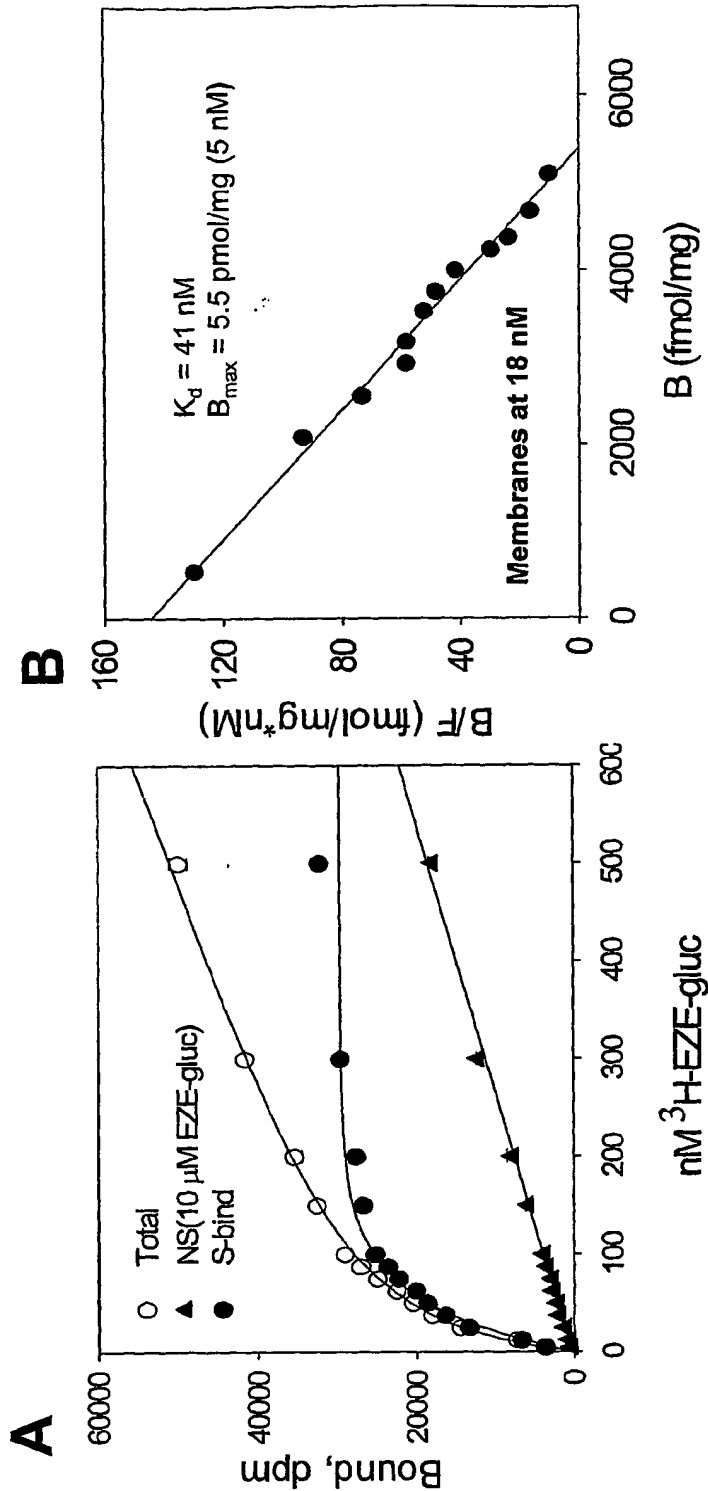
Figure 1. Equilibrium binding of EZE-glucuronide to rhesus BBMVs
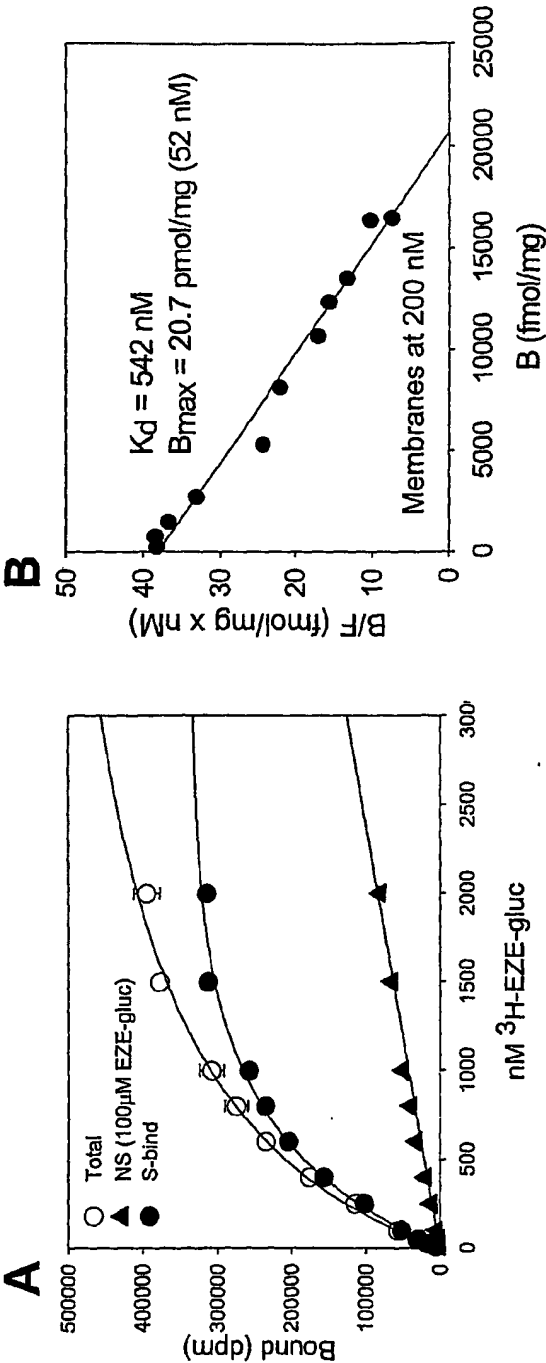
Figure 2. Equilibrium binding of EZE-glucuronide to rat BBMV.
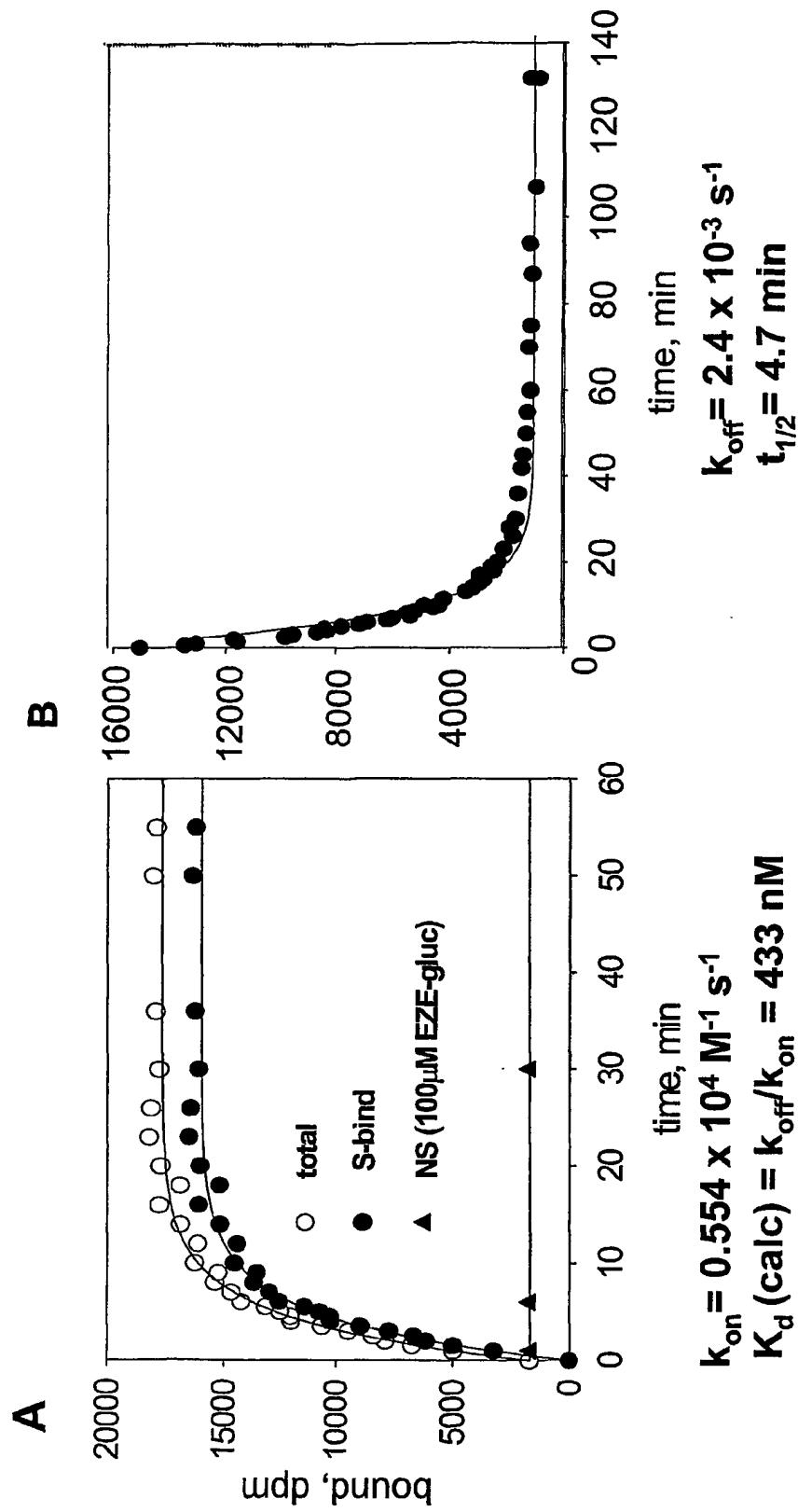
Figure 3. Association and dissociation kinetics of ^{3}H-EZE-glucuronide in rat BBMV.
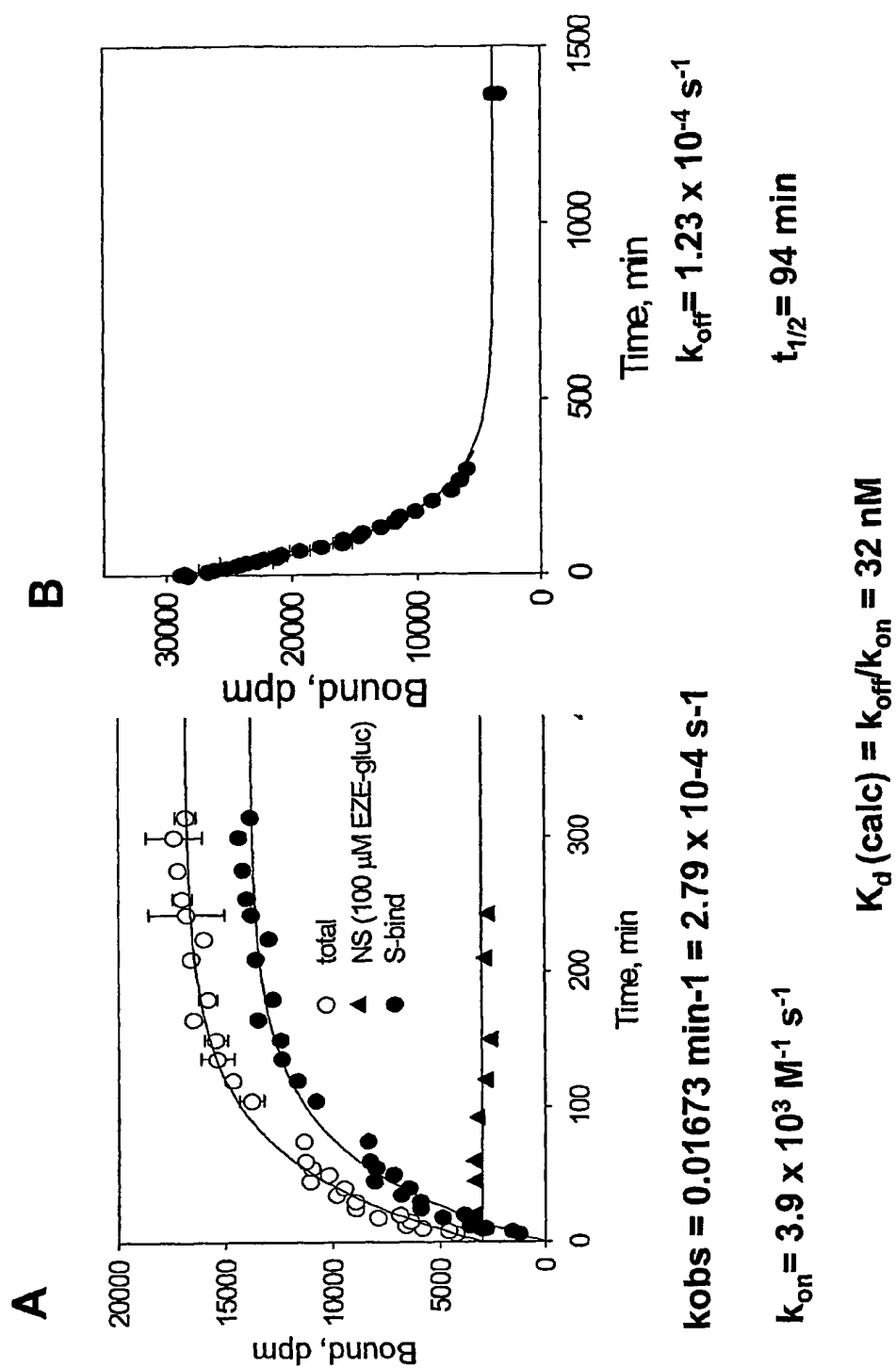
Figure 4. Association and Dissociation kinetics of ^{3}H-EZE-glucuronide in rhesus BBMV.

$k_{obs} = 0.01673$ min-1 $= 2.79 \times 10$-4 s-1

$k_{on} = 3.9 \times 10^3$ M^{-1} s^{-1}

$k_{off} = 1.23 \times 10^{-4}$ s^{-1}

$t_{1/2} = 94$ min

K_d(calc) $= k_{off}/k_{on} = 32$ nM
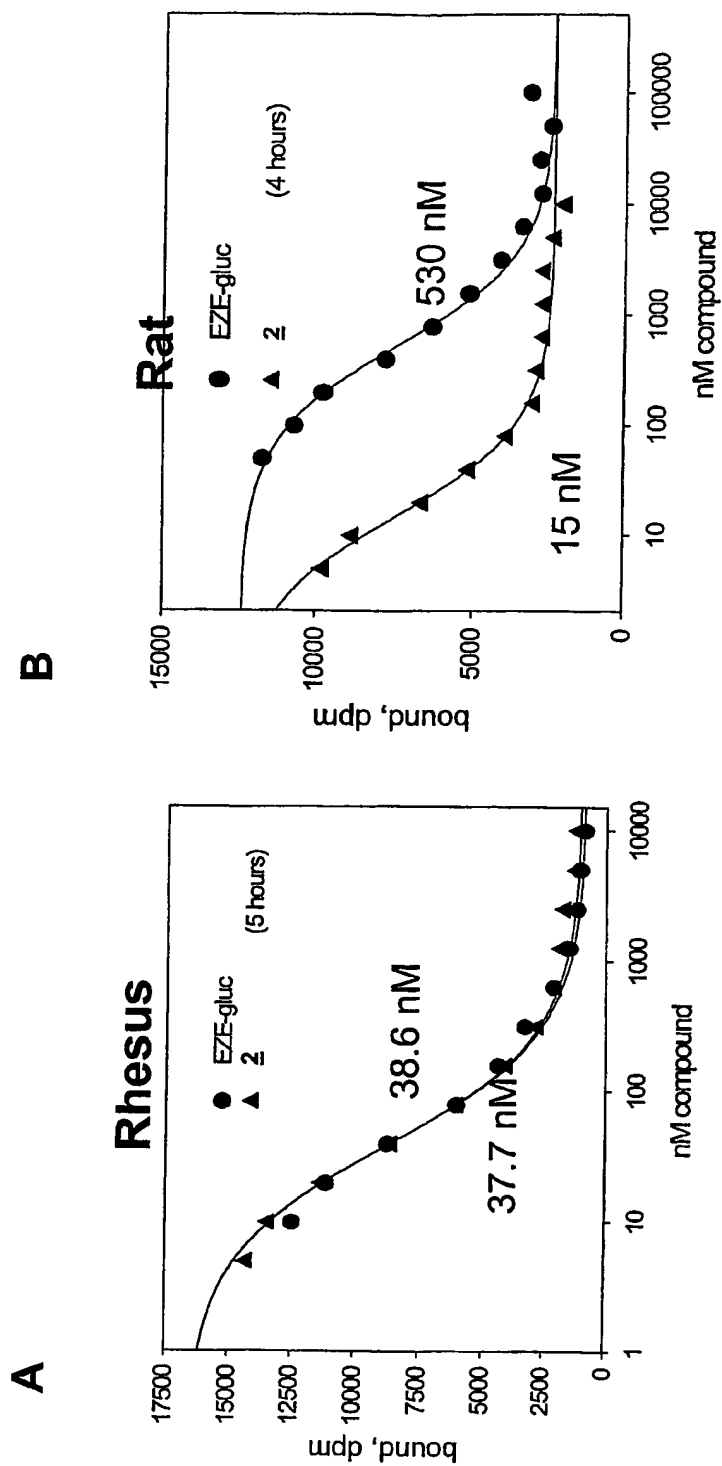
Figure 5. Displacement of 3H-EZE-glucuronide by EZE-glucuronide and compound 2 in rhesus and rat BBMV.
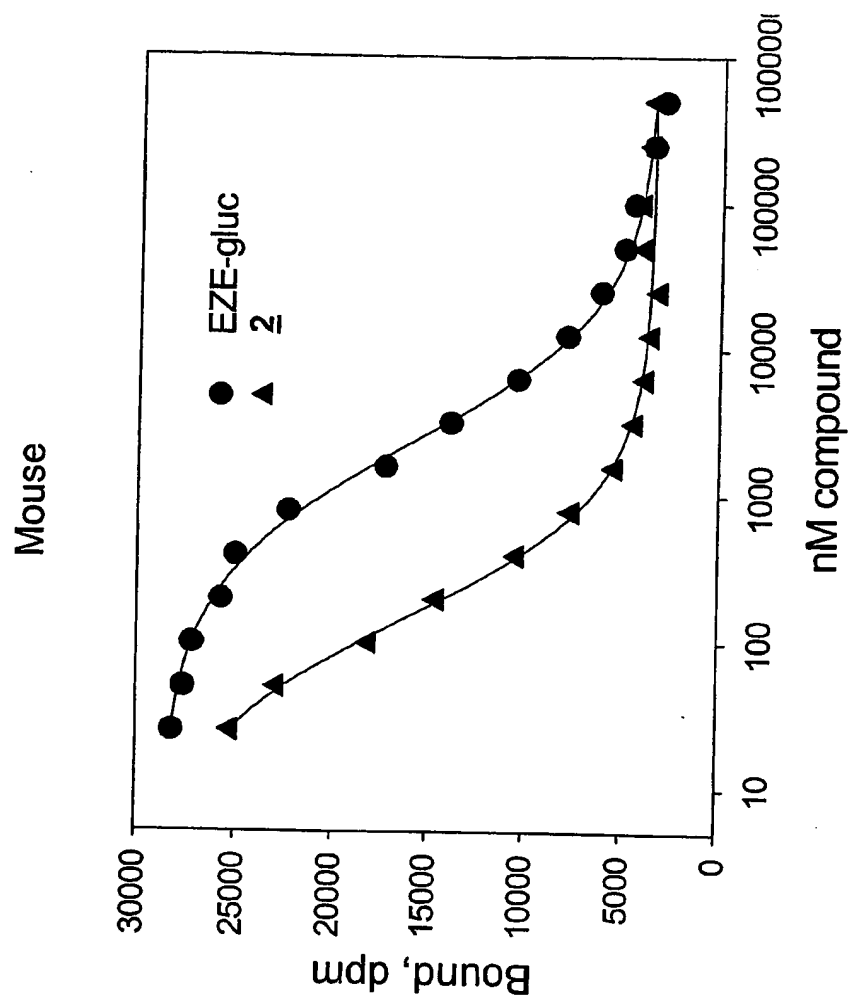
Figure 6. Displacement of ^{35}S-labeled compound **2** by EZE-glucuronide and compound **2** in mouse BBMV.
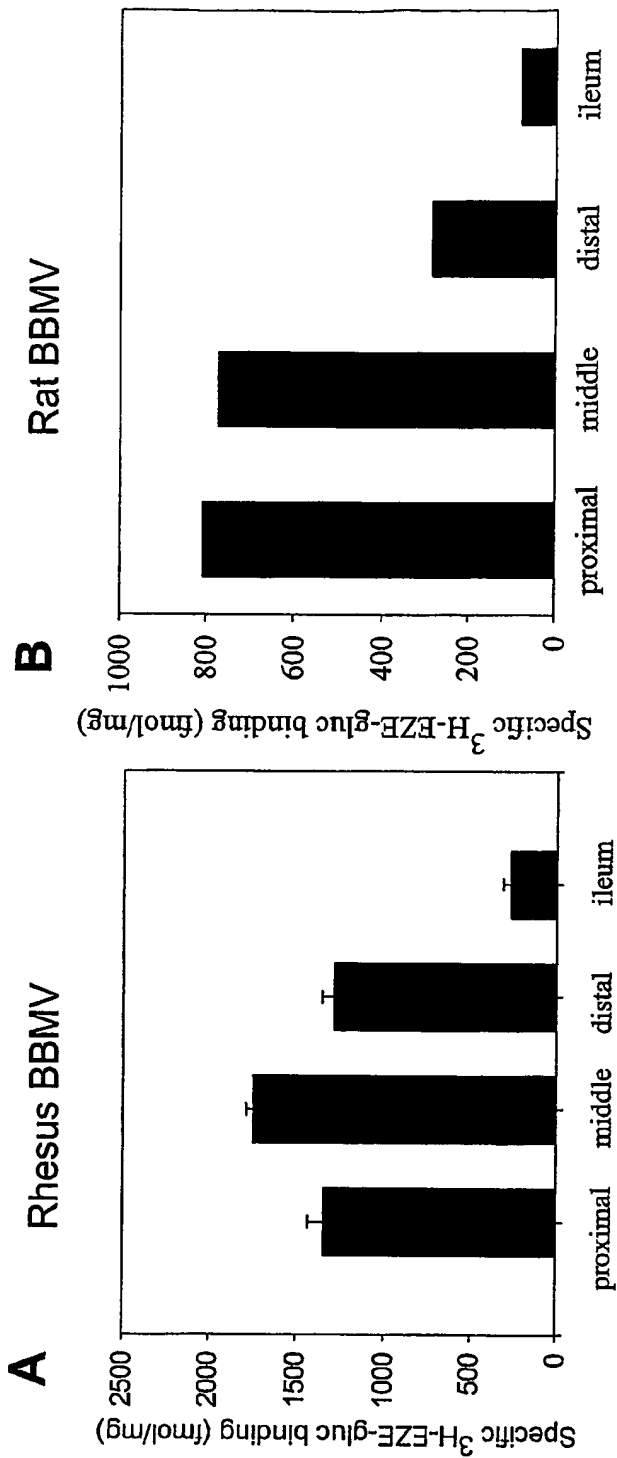
Figure 7. Intestinal distribution of ezetimibe binding sites.
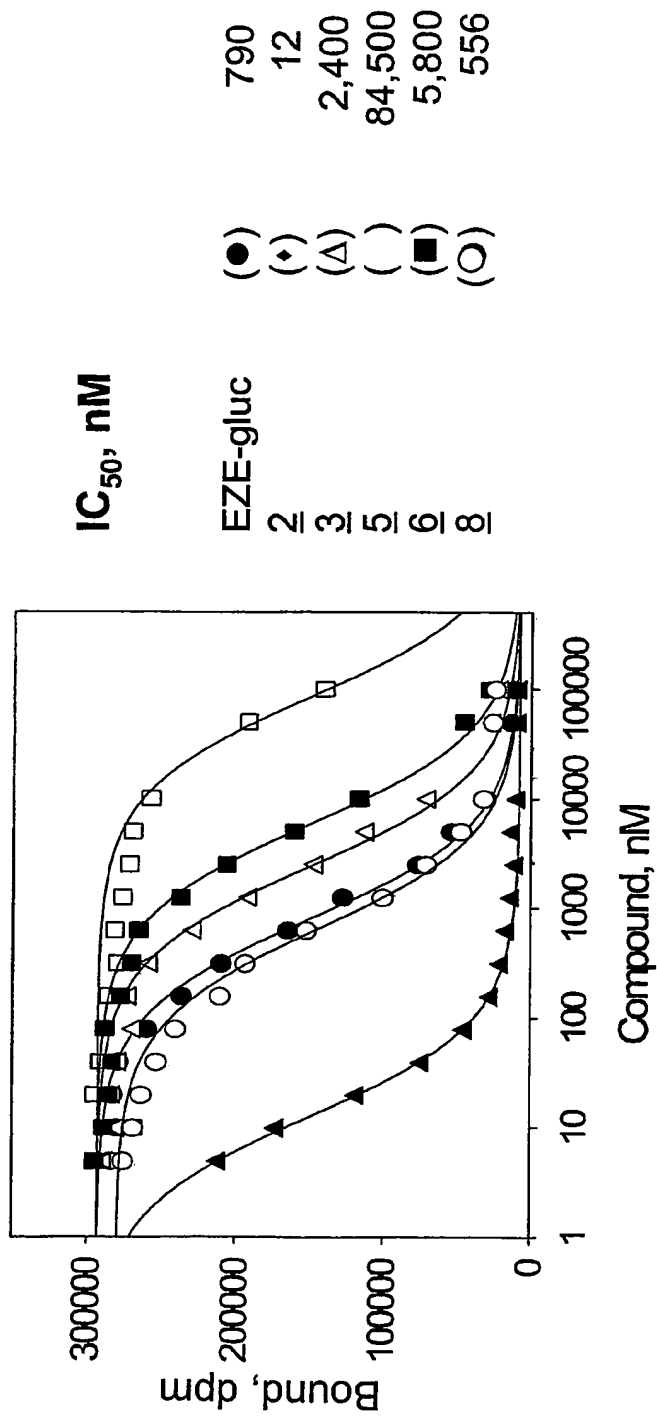
Figure 8. Displacement of ^{35}S-labeled compound 2 by EZE-glucuronide and analogs in transfected CHO cells expressing rat NPC1L1
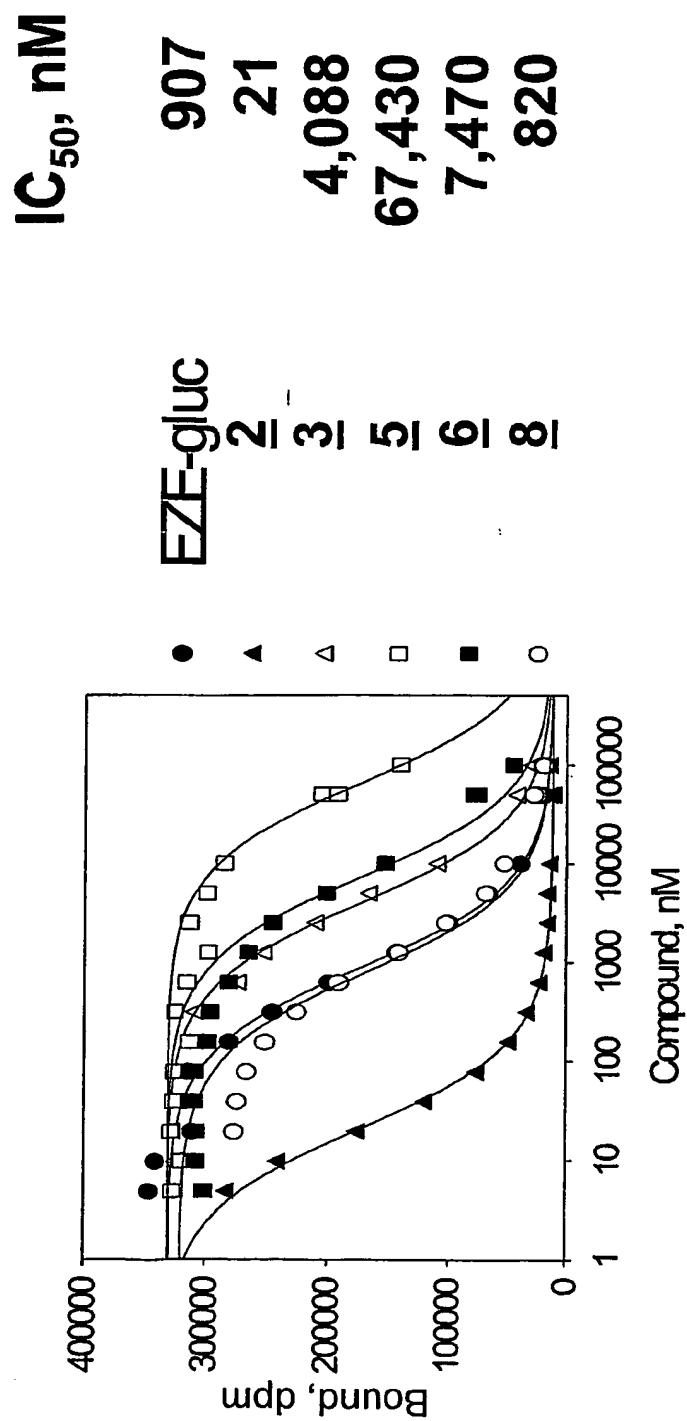
Figure 9. Displacement of ^{35}S-labeled compound 2 by EZE-glucuronide and analogs in transfected CHO cells expressing human NPC1L1
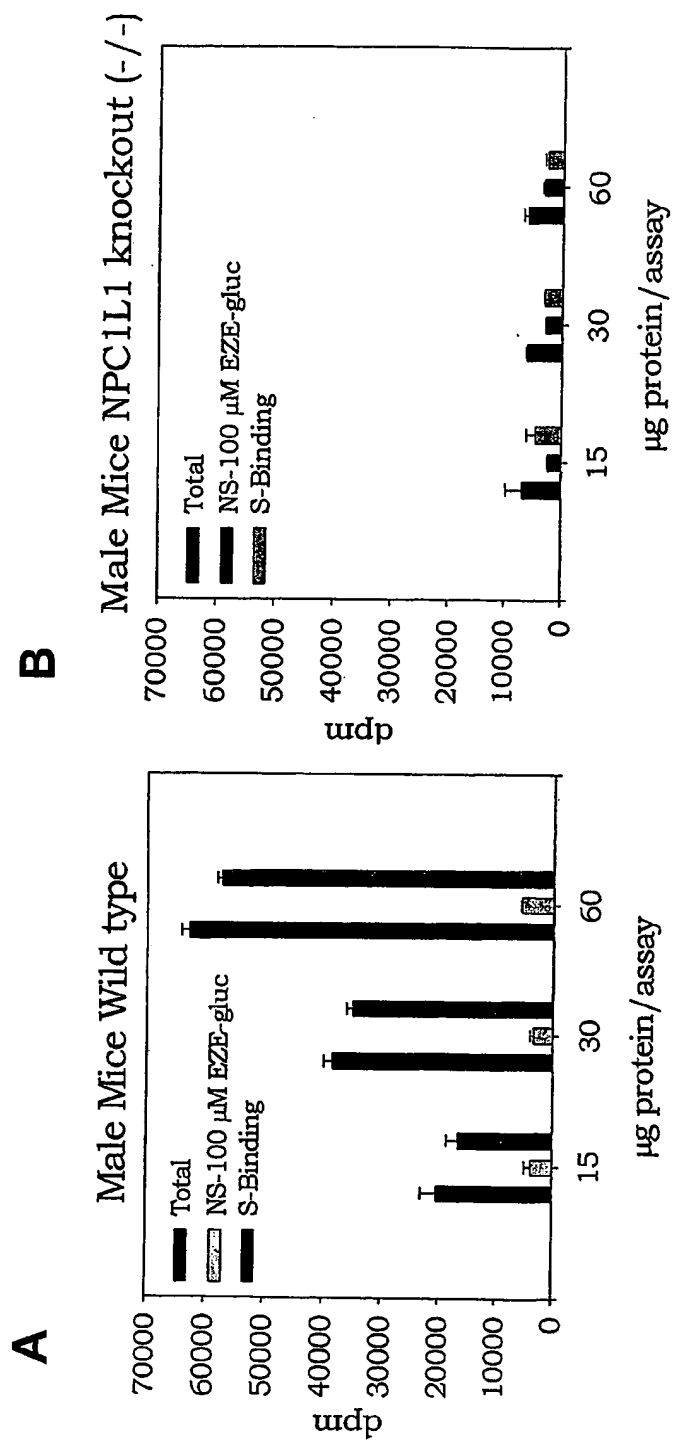
Figure 10. ^{35}S-labeled compound 2 binding with brush border membranes from intestinal mucosal scrapings of male wild type (A) and NPC1L1 knockout (-/-) mice (B).
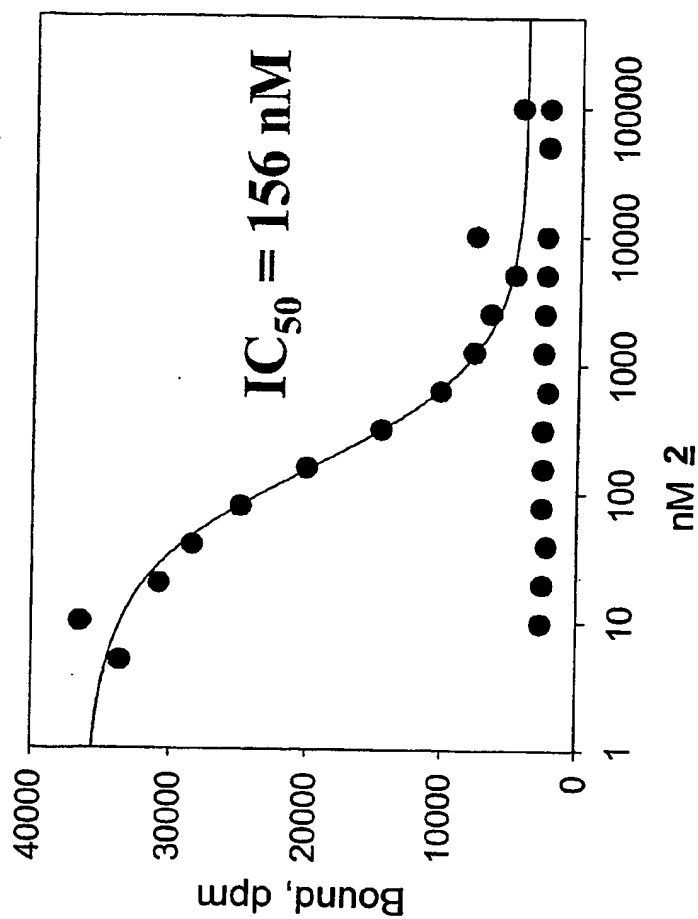
Figure 11. Displacement of ^{35}S-labeled compound 2 by compound 2 in mouse wild type and knockout mouse NPC1L1 (-/-) BBMV.

FIGURE 12

Competition

A
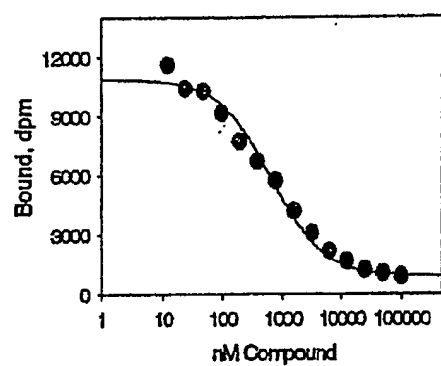
B
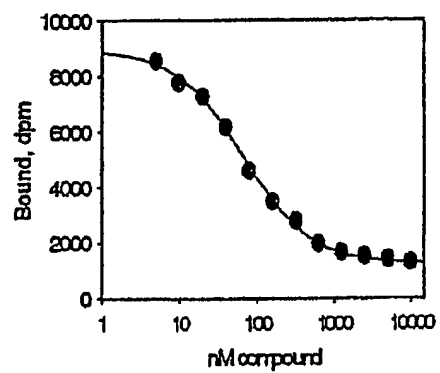
FIGURE 13

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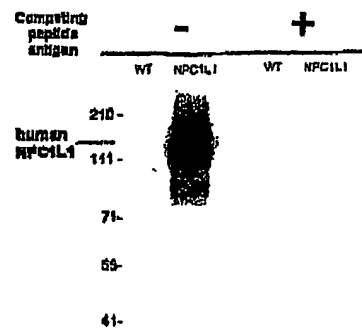
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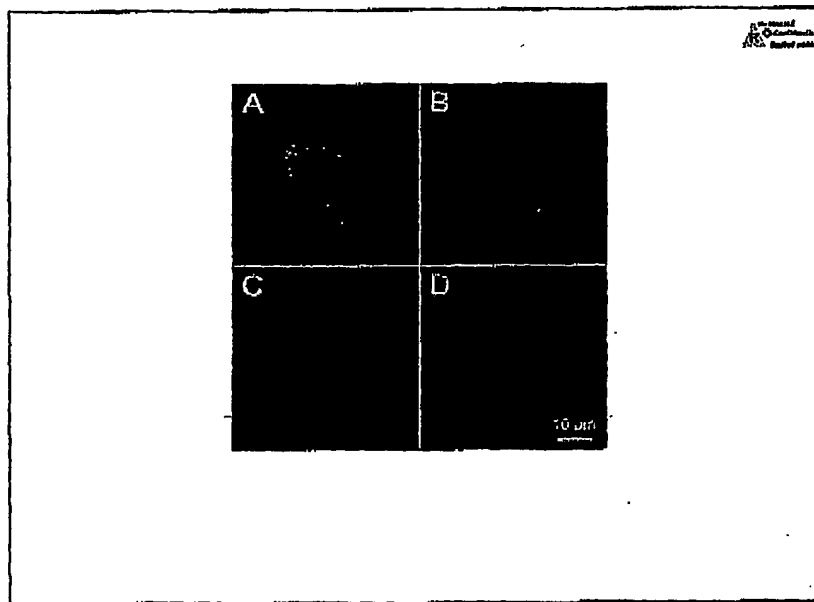
FIGURE 14
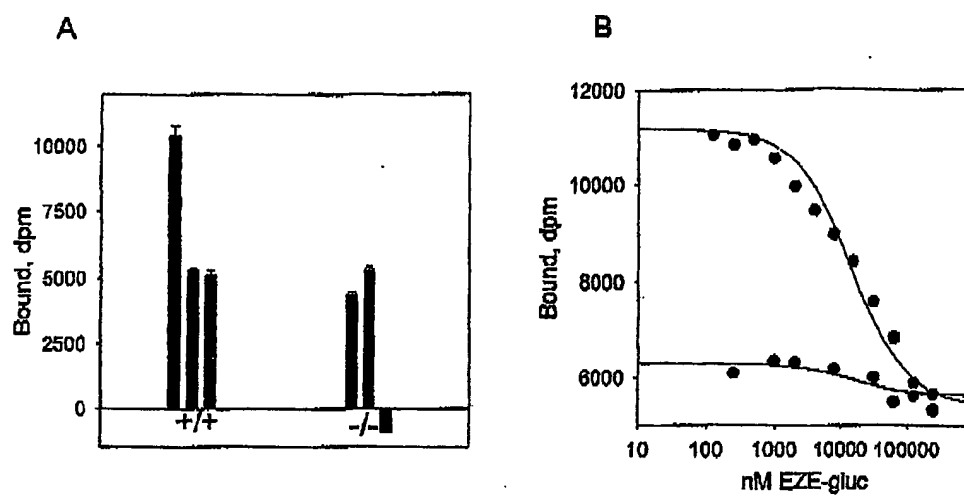
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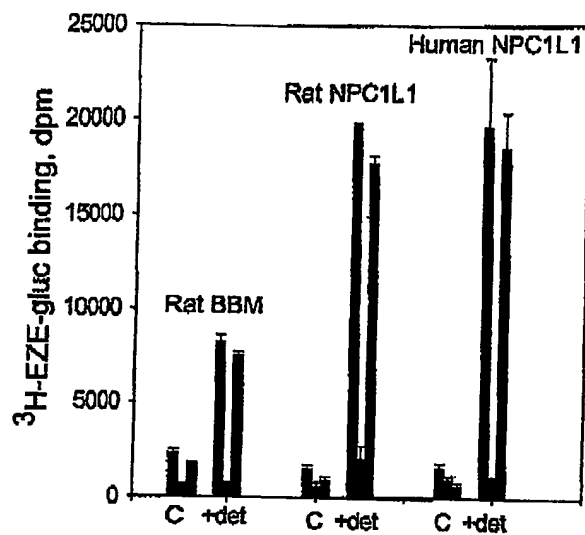
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<140> To Be Assigned

<141> 2005-01-14

<150> US 60/537,341

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                20   25   30

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	35			40			45			

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Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg
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Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr
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Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
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Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys
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        290             295             300

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305             310             315             320

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Glu Ala Pro Lys Leu Pro His Lys His Lys Leu Ser Pro His Thr Ile
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Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ser Phe His Asp Glu His
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Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Arg Asn
            405             410             415

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Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Ser Lys Asn Phe
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Ser Gly Ile Leu Ser Leu Asp Phe Leu Leu Glu Leu Leu Glu Leu Gln
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gac tac ggg gct cct gtc ttc ccc ttc ctt gct gtt ggg gga tac caa      1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
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Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp
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                965             970             975

aag gat gag ttc tgt ccc tca acg gat act tcc ttc aac tgc tta aaa    2976
Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
```

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                    980                  985                  990

aac tgc atg aac cgc act ctg ggt  cct gtg agg ccc aca  gcg gaa cag   3024
Asn Cys Met Asn Arg Thr Leu Gly  Pro Val Arg Pro Thr  Ala Glu Gln
        995                  1000                 1005

ttt cat aag tac ctg ccc tgg  ttc ctg aat gat ccg  ccc aat atc       3069
Phe His Lys Tyr Leu Pro Trp  Phe Leu Asn Asp Pro  Pro Asn Ile
    1010                 1015                 1020

aga tgt ccc aaa ggg ggt cta  gca gcg tat aga acg  tct gtg aat       3114
Arg Cys Pro Lys Gly Gly Leu  Ala Ala Tyr Arg Thr  Ser Val Asn
    1025                 1030                 1035

ttg agc tca gat ggc cag gtt  ata gcc tcc cag ttc  atg gcc tac       3159
Leu Ser Ser Asp Gly Gln Val  Ile Ala Ser Gln Phe  Met Ala Tyr
    1040                 1045                 1050

cac aag ccc tta agg aac tca  cag gac ttc aca gaa  gct ctc cgg       3204
His Lys Pro Leu Arg Asn Ser  Gln Asp Phe Thr Glu  Ala Leu Arg
    1055                 1060                 1065

gcg tcc cgg ttg cta gca gcc  aac atc aca gct gac  cta cgg aag       3249
Ala Ser Arg Leu Leu Ala Ala  Asn Ile Thr Ala Asp  Leu Arg Lys
    1070                 1075                 1080

gtg cct ggg aca gat cca aac  ttt gag gtc ttc cct  tac acg atc       3294
Val Pro Gly Thr Asp Pro Asn  Phe Glu Val Phe Pro  Tyr Thr Ile
    1085                 1090                 1095

tcc aac gtg ttc tac cag caa  tac ctg acg gtc ctt  cct gag gga       3339
Ser Asn Val Phe Tyr Gln Gln  Tyr Leu Thr Val Leu  Pro Glu Gly
    1100                 1105                 1110

atc ttc acc ctt gct ctt tgc  ttt gtg ccc acc ttt  gtt gtc tgc       3384
Ile Phe Thr Leu Ala Leu Cys  Phe Val Pro Thr Phe  Val Val Cys
    1115                 1120                 1125

tac ctc cta ctg ggc ctg gac  atg tgc tca ggg atc  ctc aac cta       3429
Tyr Leu Leu Leu Gly Leu Asp  Met Cys Ser Gly Ile  Leu Asn Leu
    1130                 1135                 1140

ctc tcc atc att atg att ctc  gtg gac acc att ggc  ctc atg gct       3474
Leu Ser Ile Ile Met Ile Leu  Val Asp Thr Ile Gly  Leu Met Ala
    1145                 1150                 1155

gtg tgg ggt atc agc tat aat  gcg gta tcc ctc atc  aac ctt gtc       3519
Val Trp Gly Ile Ser Tyr Asn  Ala Val Ser Leu Ile  Asn Leu Val
    1160                 1165                 1170

acg gca gtg ggc atg tct gtg  gag ttt gtg tcc cac  atc act cgg       3564
Thr Ala Val Gly Met Ser Val  Glu Phe Val Ser His  Ile Thr Arg
    1175                 1180                 1185

tcc ttt gct gta agc acc aag  cct acc cgg ctg gag  agg gct aaa       3609
Ser Phe Ala Val Ser Thr Lys  Pro Thr Arg Leu Glu  Arg Ala Lys
    1190                 1195                 1200

gat gct act gtc ttc atg ggc  agt gcg gtg ttt gct  gga gtg gcc       3654
```

```
      Asp Ala  Thr Val Phe Met Gly  Ser Ala Val Phe Ala  Gly Val Ala
          1205             1210             1215

      atg acc  aac ttc cca ggc atc  ctc atc ttg ggc ttt  gcc caa gcc      3699
      Met Thr  Asn Phe Pro Gly Ile  Leu Ile Leu Gly Phe  Ala Gln Ala
          1220             1225             1230

      cag ctt  att cag atc ttc ttc  ttc cgc ctc aac ctt  ctg atc acc      3744
      Gln Leu  Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
          1235             1240             1245

      ttg ctg  ggt ctg ctg cat ggc  ctg gtc ttc ctg ccg  gtt gtc ctc      3789
      Leu Leu  Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
          1250             1255             1260

      agc tat  ctg gga cca gat gtt  aac caa gct ctg gta  cag gag gag      3834
      Ser Tyr  Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Gln Glu Glu
          1265             1270             1275

      aaa cta  gcc agc gag gca gca  gtg gcc cca gag cct  tct tgc cca      3879
      Lys Leu  Ala Ser Glu Ala Ala  Val Ala Pro Glu Pro  Ser Cys Pro
          1280             1285             1290

      cag tac  ccc tcc cct gct gat  gcg gat gcc aat gtt  aac tac ggc      3924
      Gln Tyr  Pro Ser Pro Ala Asp  Ala Asp Ala Asn Val  Asn Tyr Gly
          1295             1300             1305

      ttt gcc  cca gaa ctt gcc cac  gga gct aat gct gct  aga agc tct      3969
      Phe Ala  Pro Glu Leu Ala His  Gly Ala Asn Ala Ala  Arg Ser Ser
          1310             1315             1320

      ttg ccc  aaa agt gac caa aag  ttc taa                               3996
      Leu Pro  Lys Ser Asp Gln Lys  Phe
          1325             1330
```

<210> 2

<211> 1331

<212> PRT

<213> Rattus sp.

<400> 2

```
Met Ala Ala Ala Trp Leu Gly Trp Leu Leu Trp Ala Leu Leu Leu Ser
1               5               10              15

Ala Ala Gln Gly Glu Leu Tyr Thr Pro Lys His Glu Ala Gly Val Cys
            20              25              30

Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
        35              40              45
```

```
Thr Ser Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg His
    50              55              60

Val Thr Gly Glu His Leu Ala Leu Leu Gln Arg Ile Cys Pro Arg Leu
65              70              75              80

Tyr Asn Gly Pro Asn Thr Thr Phe Ala Cys Cys Ser Thr Lys Gln Leu
                85              90              95

Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100             105             110

Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr
        115             120             125

Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Glu
    130             135             140

Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145             150             155             160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Gln Val Arg
            165             170             175

Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
        180             185             190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
    195             200             205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
210             215             220

Pro Gly Gln Ala Leu Pro Asp Gly Ile Gln Pro Leu Asn Gly Lys Ile
225             230             235             240

Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys
                245             250             255

Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Glu Ala Leu
        260             265             270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
    275             280             285
```

Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Ala Val Leu Val Arg
    290   295   300

Leu Arg Val Val Ser Asn Arg Asn Lys Asn Lys Ala Glu Gly Pro Gln
305   310   315   320

Glu Ala Pro Lys Leu Pro His Lys His Lys Leu Ser Pro His Thr Ile
            325   330   335

Leu Gly Arg Phe Phe Gln Asn Trp Gly Thr Arg Val Ala Ser Trp Pro
            340 ·   345   350

Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ala Ala
        355   360   365

Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370   375   380

Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ser Phe His Asp Glu His
385   390   395   400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Arg Asn
            405   410   415

Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Ser Lys Asn Phe
        420   425   430

Ser Gly Ile Leu Ser Leu Asp Phe Leu Leu Glu Leu Leu Glu Leu Gln
        435   440   445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Glu Arg Asn
    450   455   460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Tyr Asn Thr
465   470   475   480

Ser Leu Ser Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
            485   490   495

Asn Arg Thr Leu Leu Met Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
        500   505   510

Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala

```
                515                 520                 525


   Pro Leu Thr Phe Lys Asp Gly Thr Ser Leu Ala Leu Ser Cys Met Ala
      530                 535                 540


   Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
   545                 550                 555                 560


   Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu
                  565                 570                 575


   Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp
                  580                 585                 590


   Glu Glu Ala Phe Leu Lys Glu Met Glu Ser Phe Gln Arg Asn Thr Ser
      595                 600                 605


   Asp Lys Phe Gln Val Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
      610                 615                 620


   Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Val Ser Tyr
   625                 630                 635                 640


   Ile Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
                  645                 650                 655


   Cys Ser Arg Val Ala Val Glu Ser Lys Ala Thr Leu Gly Leu Gly Gly
                  660                 665                 670


   Val Ile Val Val Leu Gly Ala Val Leu Ala Ala Met Gly Phe Tyr Ser
                  675                 680                 685


   Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
      690                 695                 700


   Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
   705                 710                 715                 720


   Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
                  725                 730                 735


   Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
                  740                 745                 750
```

```
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
        755             760             765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe
    770             775             780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785             790             795             800

Arg Gln Glu Ala Ser Arg Pro Asp Val Leu Cys Cys Phe Ser Thr Arg
                805             810             815

Lys Leu Pro Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe
            820             825             830

Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
        835             840             845

Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met
    850             855             860

Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865             870             875             880

Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
            885             890             895

Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser
        900             905             910

Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe
        915             920             925

Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser
    930             935             940

Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945             950             955             960

Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His
            965             970             975

Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
        980             985             990
```

Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Ala Glu Gln
        995   1000   1005

Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn Ile
    1010   1015   1020

Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn
    1025   1030   1035

Leu Ser Ser Asp Gly Gln Val Ile Ala Ser Gln Phe Met Ala Tyr
    1040   1045   1050

His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg
    1055   1060   1065

Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys
    1070   1075   1080

Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile
    1085   1090   1095

Ser Asn Val Phe Tyr Gln Gln Tyr.Leu Thr Val Leu Pro Glu Gly
    1100   1105   1110

Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys
    1115   1120   1125

Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu
    1130   1135   1140

Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala
    1145   1150   1155

Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val
    1160   1165   1170

Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg
    1175   1180   1185

Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys
    1190   1195   1200

Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala
    1205   1210   1215

```
Met Thr Asn Phe Pro Gly Ile  Leu Ile Leu Gly Phe  Ala Gln Ala
    1220            1225           1230


Gln Leu Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
    1235            1240           1245


Leu Leu Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
    1250            1255           1260


Ser Tyr Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Gln Glu Glu
    1265            1270           1275


Lys Leu Ala Ser Glu Ala Ala  Val Ala Pro Glu Pro  Ser Cys Pro
    1280            1285           1290


Gln Tyr Pro Ser Pro Ala Asp  Ala Asp Ala Asn Val  Asn Tyr Gly
    1295            1300           1305


Phe Ala Pro Glu Leu Ala His  Gly Ala Asn Ala Ala  Arg Ser Ser
    1310            1315           1320


Leu Pro Lys Ser Asp Gln Lys  Phe
    1325            1330
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<210> 3

<211> 3999

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(3999)

<223>

<400> 3
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atg gcg gag gcc ggc ctg agg ggc tgg ctg ctg tgg gcc ctg ctc ctg        48
Met Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu
1               5                   10                  15
```

```
cgc ttg gcc cag agt gag cct tac aca acc atc cac cag cct ggc tac        96
Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr
        20                  25                  30

tgc gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc       144
Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
        35                  40                  45

ctc atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc       192
Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
    50                  55                  60

aag atc aca ggt gat cac ctg atc cta tta cag aag atc tgc ccc cgc       240
Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
65                  70                  75                  80

ctc tac acc ggc ccc aac acc caa gcc tgc tgc tcc gcc aag cag ctg       288
Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
                85                  90                  95

gta tca ctg gaa gcg agt ctg tcg atc acc aag gcc ctc ctc acc cgc       336
Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
        100                 105                 110

tgc cca gcc tgc tct gac aat ttt gtg aac ctg cac tgc cac aac acg       384
Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
        115                 120                 125

tgc agc ccc aat cag agc ctc ttc atc aat gtg acc cgc gtg gcc cag       432
Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
    130                 135                 140

cta ggg gct gga caa ctc cca gct gtg gtg gcc tat gag gcc ttc tac       480
Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160

cag cat agc ttt gcc gag cag agc tat gac tcc tgc agc cgt gtg cgc       528
Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
                165                 170                 175

gtc cct gca gct gcc acg ctg gct gtg ggc acc atg tgt ggc gtg tat       576
Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
        180                 185                 190

ggc tct gcc ctt tgc aat gcc cag cgc tgg ctc aac ttc cag gga gac       624
Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
        195                 200                 205

aca ggc aat ggt ctg gcc cca ctg gac atc acc ttc cac ctc ttg gag       672
Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
    210                 215                 220

cct ggc cag gcc gtg ggg agt ggg att cag cct ctg aat gag ggg gtt       720
Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
225                 230                 235                 240

gca cgt tgc aat gag tcc caa ggt gac gac gtg gcg acc tgc tcc tgc       768
Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
                245                 250                 255
```

```
caa gac tgt gct gca tcc tgt cct gcc ata gcc cgc ccc cag gcc ctc        816
Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
        260                 265                 270

gac tcc acc ttc tac ctg ggc cag atg ccg ggc agt ctg gtc ctc atc        864
Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
        275                 280                 285

atc atc ctc tgc tct gtc ttc gct gtg gtc acc atc ctg ctt gtg gga        912
Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
        290                 295                 300

ttc cgt gtg gcc ccc gcc agg gac aaa agc aag atg gtg gac ccc aag        960
Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
305                 310                 315                 320

aag ggc acc agc ctc tct gac aag ctc agc ttc tcc acc cac acc ctc       1008
Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
                325                 330                 335

ctt ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct       1056
Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
            340                 345                 350

ctg acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg       1104
Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
        355                 360                 365

ggc ctg gtc ttt aca gaa ctc act acg gac ccc gtg gag ctg tgg tcg       1152
Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370                 375                 380

gcc ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat       1200
Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
385                 390                 395                 400

ttc ggc ccc ttc ttc cga acc aac cag gtg atc ctg acg gct cct aac       1248
Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
                405                 410                 415

cgg tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc       1296
Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
                420                 425                 430

agc gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag       1344
Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
            435                 440                 445

gag agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac       1392
Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
    450                 455                 460

atc tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc       1440
Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
465                 470                 475                 480

agt ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac       1488
Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
```

```
                    485                     490                     495

aac cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag        1536
Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
            500                     505                     510

acc tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc        1584
Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
            515                     520                     525

ccg ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct        1632
Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
            530                     535                     540

gac tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa        1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
545                     550                     555                     560

gga aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc        1728
Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
                    565                     570                     575

aac aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg        1776
Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
            580                     585                     590

gag gag gcc ttc tta gag gaa atg cga gcc ttc cag cgt cgg atg gct        1824
Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
            595                     600                     605

ggc atg ttc cag gtc acg ttc acg gct gag cgc tct ctg gaa gac gag        1872
Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu
            610                     615                     620

atc aat cgc acc aca gct gaa gac ctg ccc atc ttt gcc acc agc tac        1920
Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625                     630                     635                     640

att gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc        1968
Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
                    645                     650                     655

tgg agc cga gtg atg gtg gac tcc aag gcc acg ctg ggc ctc ggc ggg        2016
Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
                    660                     665                     670

gtg gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc        2064
Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
            675                     680                     685

tac ttg ggt atc cgc tcc tcc ctg gtc atc ctg caa gtg gtt cct ttc        2112
Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
            690                     695                     700

ctg gtg ctg tcc gtg ggg gct gat aac atc ttc atc ttt gtt ctc gag        2160
Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705                     710                     715                     720

tac cag agg ctg ccc cgg agg cct ggg gag cca cga gag gtc cac att        2208
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```
Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
            725             730             735

ggg cga gcc cta ggc agg gtg gct ccc agc atg ctg ttg tgc agc ctc      2256
Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740             745             750

tct gag gcc atc tgc ttc ttc cta ggg gcc ctg acc ccc atg cca gct      2304
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
            755             760             765

gtg cgg acc ttt gcc ctg acc tct ggc ctt gca gtg atc ctt gac ttc      2352
Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
            770             775             780

ctc ctg cag atg tca gcc ttt gtg gcc ctg ctc tcc ctg gac agc aag      2400
Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785             790             795             800

agg cag gag gcc tcc cgg ttg gac gtc tgc tgc tgt gtc aag ccc cag      2448
Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
            805             810             815

gag ctg ccc ccg cct ggc cag gga gag ggg ctc ctg ctt ggc ttc ttc      2496
Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
            820             825             830

caa aag gct tat gcc ccc ttc ctg ctg cac tgg atc act cga ggt gtt      2544
Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
            835             840             845

gtg ctg ctg ctg ttt ctc gcc ctg ttc gga gtg agc ctc tac tcc atg      2592
Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
            850             855             860

tgc cac atc agc gtg gga ctg gac cag gag ctg gcc ctg ccc aag gac      2640
Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865             870             875             880

tcg tac ctg ctt gac tat ttc ctc ttt ctg aac cgc tac ttc gag gtg      2688
Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
            885             890             895

ggg gcc ccg gtg tac ttt gtt acc acc ttg ggc tac aac ttc tcc agc      2736
Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
            900             905             910

gag gct ggg atg aat gcc atc tgc tcc agt gca ggc tgc aac aac ttc      2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
            915             920             925

tcc ttc acc cag aag atc cag tat gcc aca gag ttc cct gag cag tct      2832
Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
            930             935             940

tac ctg gcc atc cct gcc tcc tcc tgg gtg gat gac ttc att gac tgg      2880
Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945             950             955             960
```

```
ctg acc ccg tcc tcc tgc tgc cgc ctt tat ata tct ggc ccc aat aag     2928
Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys
            965                 970                 975

gac aag ttc tgc ccc tcg acc gtc aac tct ctg aac tgc cta aag aac     2976
Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
            980                 985                 990

tgc atg agc atc acg atg ggc tct gtg agg ccc tcg gtg gag cag ttc     3024
Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe
            995                 1000                1005

cat aag tat ctt ccc tgg ttc ctg aac gac cgg ccc aac atc aaa         3069
His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys
        1010            1015                1020

tgt ccc aaa ggc ggc ctg gca gca tac agc acc tct gtg aac ttg         3114
Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu
    1025                1030                1035

act tca gat ggc cag gtt tta gcc tcc agg ttc atg gcc tat cac         3159
Thr Ser Asp Gly Gln Val Leu Ala Ser Arg Phe Met Ala Tyr His
    1040                1045                1050

aag ccc ctg aaa aac tca cag gat tac aca gaa gct ctg cgg gca         3204
Lys Pro Leu Lys Asn Ser Gln Asp Tyr Thr Glu Ala Leu Arg Ala
    1055                1060                1065

gct cga gag ctg gca gcc aac atc act gct gac ctg cgg aaa gtg         3249
Ala Arg Glu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys Val
    1070                1075                1080

cct gga aca gac ccg gct ttt gag gtc ttc ccc tac acg atc acc         3294
Pro Gly Thr Asp Pro Ala Phe Glu Val Phe Pro Tyr Thr Ile Thr
    1085                1090                1095

aat gtg ttt tat gag cag tac ctg acc atc ctc cct gag ggg ctc         3339
Asn Val Phe Tyr Glu Gln Tyr Leu Thr Ile Leu Pro Glu Gly Leu
    1100                1105                1110

ttc atg ctc agc ctc tgc ctt gtg ccc acc ttc gct gtc tcc tgc         3384
Phe Met Leu Ser Leu Cys Leu Val Pro Thr Phe Ala Val Ser Cys
    1115                1120                1125

ctc ctg ctg ggc ctg gac ctg cgc tcc ggc ctc ctc aac ctg ctc         3429
Leu Leu Leu Gly Leu Asp Leu Arg Ser Gly Leu Leu Asn Leu Leu
    1130                1135                1140

tcc att gtc atg atc ctc gtg gac act gtc ggc ttc atg gcc ctg         3474
Ser Ile Val Met Ile Leu Val Asp Thr Val Gly Phe Met Ala Leu
    1145                1150                1155

tgg gac atc agt tac aat gct gtg tcc ctc atc aac ctg gtc tcg         3519
Trp Asp Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val Ser
    1160                1165                1170

gcg gtg ggc atg tct gtg gag ttt gtg tcc cac att acc cgc tcc         3564
Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg Ser
    1175                1180                1185
```

```
ttt gcc atc agc acc aag ccc  acc tgg ctg gag agg  gcc aaa gag       3609
Phe Ala Ile Ser Thr Lys Pro  Thr Trp Leu Glu Arg  Ala Lys Glu
    1190            1195               1200

gcc acc atc tct atg gga agt  gcg gtg ttt gca ggt  gtg gcc atg       3654
Ala Thr Ile Ser Met Gly Ser  Ala Val Phe Ala Gly  Val Ala Met
    1205            1210               1215

acc aac ctg cct ggc atc ctt  gtc ctg ggc ctc gcc  aag gcc cag       3699
Thr Asn Leu Pro Gly Ile Leu  Val Leu Gly Leu Ala  Lys Ala Gln
    1220            1225               1230

ctc att cag atc ttc ttc ttc  cgc ctc aac ctc ctg  atc act ctg       3744
Leu Ile Gln Ile Phe Phe Phe  Arg Leu Asn Leu Leu  Ile Thr Leu
    1235            1240               1245

ctg ggc ctg ctg cat ggc ttg  gtc ttc ctg ccc gtc  atc ctc agc       3789
Leu Gly Leu Leu His Gly Leu  Val Phe Leu Pro Val  Ile Leu Ser
    1250            1255               1260

tac gtg ggg cct gac gtt aac  ccg gct ctg gca ctg  gag cag aag       3834
Tyr Val Gly Pro Asp Val Asn  Pro Ala Leu Ala Leu  Glu Gln Lys
    ·1265           1270               1275

cgg gct gag gag gcg gtg gca  gca gtc atg gtg gcc  tct tgc cca       3879
Arg Ala Glu Glu Ala Val Ala  Ala Val Met Val Ala  Ser Cys Pro
    1280            1285               1290

aat cac ccc tcc cga gtc tcc  aca gct gac aac atc  tat gtc aac       3924
Asn His Pro Ser Arg Val Ser  Thr Ala Asp Asn Ile  Tyr Val Asn
    1295            1300               1305

cac agc ttt gaa ggt tct atc  aaa ggt gct ggt gcc  atc agc aac       3969
His Ser Phe Glu Gly Ser Ile  Lys Gly Ala Gly Ala  Ile Ser Asn
    1310            1315               1320

ttc ttg ccc aac aat ggg cgg  cag ttc tga                            3999
Phe Leu Pro Asn Asn Gly Arg  Gln Phe
    1325            1330
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<210> 4

<211> 1332

<212> PRT

<213> Homo sapiens

<400> 4

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Met Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu
1               5                   10                  15

Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr
```

```
                  20                      25                      30

       Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
            35                  40                  45


       Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
            50                  55                  60


       Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
       65                  70                  75                  80


       Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
                        85                  90                  95


       Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
                    100                 105                 110


       Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
                115                 120                 125


       Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
            130                 135                 140


       Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
       145                     150                 155                 160


       Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
                        165                 170                 175


       Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
                    180                 185                 190


       Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
                195                 200                 205


       Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
            210                 215                 220


       Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
       225                 230                 235                 240


       Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
                        245                 250                 255
```

Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
        260   265   270

Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
     275   280   285

Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
     290   295   300

Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
305   310   315   320

Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
          325   330   335

Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
        340   345   350

Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
       355   360   365

Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
   370   375   380

Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
385   390   395   400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
        405   410   415

Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
        420   425   430

Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
       435   440   445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
   450   455   460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
465   470   475   480

Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
        485   490   495

```
Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
        500             505                 510


Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515             520                 525


Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
        530             535                 540


Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
545             550             555                 560


Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
            565             570                 575


Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
            580             585                 590


Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
        595             600                 605


Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu
    610             615                 620


Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625             630             635                 640


Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
            645             650                 655


Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
            660             665                 670


Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
        675             680                 685


Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
    690             695                 700


Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705             710             715                 720


Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
            725             730                 735
```

Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740   745   750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
            755   760   765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
    770   775   780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785   790   795   800

Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
                805   810   815

Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
            820   825   830

Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
            835   840   845

Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
    850   855   860

Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865   870   .   875   880

Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
            885   890   895

Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
            900   905   910

Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
            915   920   925

Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
    930   935   940

Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945   950   955   960

Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys

```
                        965                 970                 975


     Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
                 980                 985                 990


     Cys Met Ser Ile Thr Met Gly Ser  Val Arg Pro Ser Val  Glu Gln Phe
             995                 1000                1005


     His Lys Tyr Leu Pro Trp Phe  Leu Asn Asp Arg Pro  Asn Ile Lys
         1010                1015                1020


     Cys Pro Lys Gly Gly Leu Ala  Ala Tyr Ser Thr Ser  Val Asn Leu
         1025                1030                1035


     Thr Ser Asp Gly Gln Val Leu  Ala Ser Arg Phe Met  Ala Tyr His
         1040                1045                1050


     Lys Pro Leu Lys Asn Ser Gln  Asp Tyr Thr Glu Ala  Leu Arg Ala
         1055                1060                1065


     Ala Arg Glu Leu Ala Ala Asn  Ile Thr Ala Asp Leu  Arg Lys Val
         1070                1075                1080


     Pro Gly Thr Asp Pro Ala Phe  Glu Val Phe Pro Tyr  Thr Ile Thr
         1085                1090                1095


     Asn Val Phe Tyr Glu Gln Tyr  Leu Thr Ile Leu Pro  Glu Gly Leu
         1100                1105                1110


     Phe Met Leu Ser Leu Cys Leu  Val Pro Thr Phe Ala  Val Ser Cys
         1115                1120                1125


     Leu Leu Leu Gly Leu Asp Leu  Arg Ser Gly Leu Leu  Asn Leu Leu
         1130                1135                1140


     Ser Ile Val Met Ile Leu Val  Asp Thr Val Gly Phe  Met Ala Leu
         1145                1150                1155


     Trp Asp Ile Ser Tyr Asn Ala  Val Ser Leu Ile Asn  Leu Val Ser
         1160                1165                1170


     Ala Val Gly Met Ser Val Glu  Phe Val Ser His Ile  Thr Arg Ser
         1175                1180                1185
```

```
Phe Ala  Ile Ser Thr Lys Pro  Thr Trp Leu Glu Arg  Ala Lys Glu
    1190                 1195              1200
```

```
Ala Thr  Ile Ser Met Gly Ser  Ala Val Phe Ala Gly  Val Ala Met
    1205                 1210              1215
```

```
Thr Asn  Leu Pro Gly Ile Leu  Val Leu Gly Leu Ala  Lys Ala Gln
    1220                 1225              1230
```

```
Leu Ile  Gln Ile Phe Phe Phe  Arg Leu Asn Leu Leu  Ile Thr Leu
    1235                 1240              1245
```

```
Leu Gly  Leu Leu His Gly Leu  Val Phe Leu Pro Val  Ile Leu Ser
    1250                 1255              1260
```

```
Tyr Val  Gly Pro Asp Val Asn  Pro Ala Leu Ala Leu  Glu Gln Lys
    1265                 1270              1275
```

```
Arg Ala  Glu Glu Ala Val Ala  Ala Val Met Val Ala  Ser Cys Pro
    1280                 1285              1290
```

```
Asn His  Pro Ser Arg Val Ser  Thr Ala Asp Asn Ile  Tyr Val Asn
    1295                 1300              1305
```

```
His Ser  Phe Glu Gly Ser Ile  Lys Gly Ala Gly Ala  Ile Ser Asn
    1310                 1315              1320
```

```
Phe Leu  Pro Asn Asn Gly Arg  Gln Phe
    1325             .    1330
```

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<210>  5

<211>  885

<212>  DNA

<213>  Rattus sp.
```

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acatctgcta tgcccccctc aacccatata acaccagcct ctccgactgc tgtgtcaaca     120

gcctccttca gtacttccag aacaaccgca ccctcctgat gctcacggcc aaccagactc     180

tgaatggcca gacctccctg gtggactgga aggaccattt cctctactgt gcaaatgccc     240
```

```
ctctcacgtt caaagatggc acgtctctgg ccctgagctg catggctgac tacggggctc    300

ctgtcttccc cttccttgct gttggggggat accaaggcac ggactattcc gaggcagaag    360

cgctgatcat aaccttctct ctcaataact accccgctga tgatccccgc atggcccagg    420

ccaagctctg ggaggaggct ttcttgaagg aaatggaatc cttccagagg aacacaagtg    480

acaagttcca ggttgcgttc tcagctgagc gctctctgga ggatgagatc aaccgcacca    540

ccatccagga cctgcctgtc tttgccgtca gctacattat cgtcttcctg tacatctccc    600

tggccctggg cagctactcc agatgcagcc gagtagcggt ggagtccaag gctactctgg    660

gcctaggtgg ggtgatagtg tgctgggagc agttctggct tgcatggggc ttctaactcc    720

tacctgggtg tcccctcttc tctggttatc atccaagtgg tacctttcct ggtgcttaag    780

ctgtgggagc tggacacatc tacatcctag acttgagtac cagaggtacc taggaagccg    840

cggaacagcg aaaaggacac attgggcgca ccctgggcat gtggc              885
```

<210> 6

<211> 458

<212> DNA

<213> Rattus sp.

<400> 6
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gaccagatgt taaccaagct ctggtacagg aggagaaact agccagcgag gcagcagtgg    60

ccccagagcc ttcttgccca cagtaccccct cccctgctga tgcggatgcc aatgttaact    120

acggctttgc cccagaactt gcccacggag ctaatgctgc tagaagctct ttgcccaaaa    180

gtgaccaaaa gttctaatgg agtaggagct tgtccatgct tctgctgatg agggatcatg    240

aaggtcttcc ctctggttgt cctcaaggcc tggggggagg ttgttcagag aaaaatggct    300

ggcattcctg ccacgaggca accggcagct tggcactgac tccttggtct cataggtccc    360

taaggcttgg tcagattact cctcatggag agactatctt aagtatctaa gctatcgatt    420

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```

<210> 7

<211> 896

<212> DNA

<213> Rattus sp.

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gatgtcccaa aggggggtcta gcagcgtata gaacgtctgt gaatttgagc tcagatggcc       120

aggttatagc ctcccagttc atggcctacc acaagccctt aaggaactca caggacttca       180

cagaagctct ccgggcgtcc cggttgctag cagccaacat cacagctgac ctacggaagg       240

tgcctgggac agatccaaac tttgaggtct tcccttacac gatctccaac gtgttctacc       300

agcaatacct gacggtcctt cctgagggaa tcttcaccct tgctctttgc tttgtgccca       360

cctttgttgt ctgctacctc ctactgggcc tggacatgtg ctcagggatc ctcaacctac       420

tctccatcat tatgattctc gtggacacca ttggcctcat ggctgtgtgg ggtatcagct       480

ataatgcggt atccctcatc aaccttgtca cggcagtggg catgtctgtg gagtttgtgt       540

cccacatcac tcggtccttt gcttgtaagc accaagccta cccggctgga gagggctaaa       600

agatgctact gtcttcatgg gcagtgcggt gtttgctgga gtggccatga ccaacttccc       660

aggcatcctc atcttggggg ctttgcccca agcccaggct tattcagatc ttcttcttcc       720

gcctcaacct tctgatcacc tttgctgggg tctgctgcat ggctggtctt cctgcccggt       780

ttgtcctcag ctatctggga ccagatgtaa ccaaggctct gctacccgga ggagaaacta       840

gccagcgagg gcagcagtgg ccccagagac ttcttgccca caagtaccct tccctg         896
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<210>  8

<211>  3124

<212>  DNA

<213>  Rattus sp.
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tccagaacaa ccgcaccctc ctgatgctca cggccaacca gactctgaat ggccagacct       180

ccctggtgga ctggaaggac catttcctct actgtgcaaa tgcccctctc acgttcaaag       240

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ttgctgttgg gggataccaa ggcacggact attccgaggc agaagcgctg atcataacct       360

tctctctcaa taactacccc gctgatgatc ccgcatggc ccaggccaag ctctgggagg       420
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aggctttctt gaaggaaatg gaatccttcc agaggaacac aagtgacaag ttccaggttg    480

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ctgtctttgc cgtcagctac attatcgtct tcctgtacat ctccctggcc ctgggcagct    600

actccagatg cagccgagta gcggtggagt ccaaggctac tctgggccta ggtggggtga    660

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cttctctggt tatcatccaa gtggtacctt tcctggtgct agctgtggga gctgacaaca    780

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ctcacattgg ccgcaccctg ggcagtgtgg cccccagcat gctgctgtgc agcctctctg    900

aggccatctg cttctttcta ggggccctga cccccatgcc agctgtgagg accttcgcct    960

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tgaccctgtt tggagcaaat ctctacttaa tgtgcaacat caacgtgggg ctagaccagg   1260

agctggctct gcccaaggac tcgtacttga tagactactt cctctttctg aaccgatacc   1320

ttgaagtggg gcctccagtg tactttgtca ccacctcggg cttcaacttc tccagcgagg   1380

caggcatgaa cgccacttgc tctagcgcag ctgtaagag cttctcccta acccagaaaa     1440

tccagtatgc cagtgaattc cctgaccagt cttacgtggc tattgctgca tcctcctggg   1500

tagatgactt catcgactgg ctgaccccgt cctcctcctg ctgtcgcctt tatatacgtg   1560

gcccccataa ggatgagttc tgtccctcaa cggatacttc cttcaactgc ttaaaaaact   1620

gcatgaaccg cactctgggt cctgtgaggc ccacagcgga acagtttcat aagtacctgc   1680

cctggttcct gaatgatccg cccaatatca gatgtcccaa agggggtcta gcagcgtata   1740

gaacgtctgt gaatttgagc tcagatggcc aggttatagc ctcccagttc atggcctacc   1800

acaagccctt aaggaactca caggacttca cagaagctct ccgggcgtcc cggttgctag   1860

cagccaacat cacagctgac ctacggaagg tgcctgggac agatccaaac tttgaggtct   1920

tcccttacac gatctccaac gtgttctacc agcaatacct gacggtcctt cctgagggaa   1980

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```

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ccaagcctac ccggctggag agggctaaag atgctactgt cttcatgggc agtgcggtgt    2280

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agcttattca gatcttcttc ttccgcctca accttctgat caccttgctg ggtctgctgc    2400

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cccacggagc taatgctgct agaagctctt tgcccaaaag tgaccaaaag ttctaatgga    2640

gtaggagctt gtccatgctt cttgctgatg agggatcatg aaggtcttcc ctctggttgt    2700

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gggcaatcag ccatgtggca gaatgtaaat taatataaat gggttgtctt aagttatgat    3060

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<210> 9

<211> 4484

<212> DNA

<213> Rattus sp.

<400> 9

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ccggcccgcc acgtcacggg tgaacacctg gctcttctcc agcgcatctg tcccgcctg     240

tacaacggcc ccaataccac ttttgcctgt tgctctacca agcagctgct gtccttagaa    300

agcagcatgt ccatcaccaa ggcccttctc acgcgctgcc cggcctgctc tgacaatttt    360

gtgagcttac actgccacaa cacttgcagc cctgaccaga gcctcttcat caacgtcacc    420
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cgggtggttg agcggggcgc tggagagcct cctgccgtgg tggcctatga ggccttttat    480

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gcttccttgg ccgtgggcag catgtgtgga gtgtatggct ccgccctctg caatgctcag    600

cgctggctca acttccaagg agacacaggg aatggcctgg ctccgctgga tatcaccttc    660

cacctcttgg agcctggcca ggccctaccg gatgggatcc agccactgaa tgggaagatc    720

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atcgttgtga tagccttagc agcaggcctg acctttattg aactcaccac agaccctgtg    1140

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cctatggctc  aggcagggcc  atccggaaga  agagaggatt  ctgggataaa  gccaggtggg      4320

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gaatgtaaat  taatataaat  gggttgtctt  aagttatgat  tctagctggg  gaggagccta      4440

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<210> 10

<211> 3993
<212> DNA

<213> Rattus sp.

<220>

<221> misc_feature

<222> (1)..(3993)

<223> n is g or a or t or c

<400> 10

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garytntaya  cnccnaarca  ygargcnggn  gtntgyacnt  tytaygarga  rtgyggnaar      120

aayccngary  tnwsnggngg  nytnacnwsn  ytnwsnaayg  tnwsntgyyt  nwsnaayacn      180

ccngcnmgnc  aygtnacngg  ngarcayytn  gcnytnytnc  armgnathtg  yccnmgnytn      240

tayaayggnc  cnaayacnac  nttygcntgy  tgywsnacna  arcarytnyt  nwsnytngar      300

wsnwsnatgw  snathacnaa  rgcnytnytn  acnmgntgyc  cngcntgyws  ngayaaytty      360

gtnwsnytnc  aytgycayaa  yacntgywsn  ccngaycarw  snytnttyat  haaygtnacn      420

mgngtngtng  armgnggngc  nggngarccn  ccngcngtng  tngctayga  rgcnttytay      480

carmgnwsnt  tygcngaraa  rgcntaygar  wsntgywsnc  argtnmgnat  hccngcngcn      540

gcnwsnytng  cngtnggnws  natgtgyggn  gtntayggnw  sngcnytntg  yaaygcncar      600

mgntggytna  ayttycargg  ngayacnggn  aayggnytng  cnccnytnga  yathacntty      660

cayytnytng  arccnggnca  rgcnytnccn  gayggnathc  arccnytnaa  yggnaarath      720
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gcnccntgya aygarwsnca rggngaygay wsngcngtnt gywsntgyca rgaytgygcn      780

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gtnytngtnm gnytnmgngt ngtnwsnaay mgnaayaara ayaargcnga rggnccncar      960

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ttycaraayt ggggnacnmg ngtngcnwsn tggccnytna cngtnytngc nytnwsntty     1080

athgtngtna thgcnytngc ngcnggnytn acnttyathg arytnacnac ngayccngtn     1140

garytntggw sngcnccnaa rwsncargcn mgnaargara arwsnttyca ygaygarcay     1200

ttyggnccnt tyttymgnac naaycarath ttygtnacng cnmgnaaymg nwsnwsntay     1260

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gcngarmgna ayathwsnyt ncargayath tgytaygcnc cnytnaaycc ntayaayacn     1440

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ytnatgytna cngcnaayca racnytnaay ggncaracnw snytngtnga ytggaargay     1560

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garwsnttyc armgnaayac nwsngayaar ttycargtng cnttywsngc ngarmgnwsn     1860

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athathgtnt tyytntayat hwsnytngcn ytnggnwsnt aywsnmgntg ywsnmgngtn     1980

gcngtngarw snaargcnac nytnggnytn ggnggngtna thgtngtnyt nggngcngtn     2040

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<210> 11

<211> 4002

<212> DNA

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<213>   Mus sp.


<220>

<221>   CDS

<222>   (1)..(4002)

<223>


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Met Ala Ala Ala Trp Gln Gly Trp Leu Leu Trp Ala Leu Leu Leu Asn
1               5                   10                  15


tcg gcc cag ggt gag ctc tac aca ccc act cac aaa gct ggc ttc tgc        96
Ser Ala Gln Gly Glu Leu Tyr Thr Pro Thr His Lys Ala Gly Phe Cys
                20                  25                  30


acc ttt tat gaa gag tgt ggg aag aac cca gag ctt tct gga ggc ctc       144
Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
            35                  40                  45


aca tca cta tcc aat atc tcc tgc ttg tct aat acc cca gcc cgc cat       192
Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His
        50                  55                  60


gtc aca ggt gac cac ctg gct ctt ctc cag cgc gtc tgt ccc cgc cta       240
Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu
65                  70                  75                  80


tac aat ggc ccc aat gac acc tat gcc tgt tgc tct acc aag cag ctg       288
Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu
                85                  90                  95


gtg tca tta gac agt agc ctg tct atc acc aag gcc ctc ctt aca cgc       336
Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
                100                 105                 110


tgc ccg gca tgc tct gaa aat ttt gtg agc ata cac tgt cat aat acc       384
Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr
            115                 120                 125


tgc agc cct gac cag agc ctc ttc atc aat gtt act cgc gtg gtt cag       432
Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln
        130                 135                 140


cgg gac cct gga cag ctt cct gct gtg gtg gcc tat gag gcc ttt tat       480
Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160


caa cgc agt ttt gca gag aag gcc tat gag tcc tgt agc cgg gtg cgc       528
Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg
                165                 170                 175
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
        180                 185                 190

ggc tct gcc ctc tgc aat gct cag cgc tgg ctc aac ttc caa gga gac       624
Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
        195                 200                 205

aca ggg aat ggc ctg gct ccg ctg gac atc acc ttc cac ctc ttg gag       672
Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
        210                 215                 220

cct ggc cag gcc ctg gca gat ggg atg aag cca ctg gat ggg aag atc       720
Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile
225                 230                 235                 240

aca ccc tgc aat gag tcc cag ggt gaa gac tcg gca gcc tgt tcc tgc       768
Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys
                245                 250                 255

cag gac tgt gca gca tcc tgc cct gtc atc cct ccg ccc ccg gcc ctg       816
Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Pro Ala Leu
        260                 265                 270

cgc cct tct ttc tac atg ggt cga atg cca ggc tgg ctg gct ctc atc       864
Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
        275                 280                 285

atc atc ttc act gct gtc ttt gta ttg ctc tct gtt gtc ctt gtg tat       912
Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr
        290                 295                 300

ctc cga gtg gct tcc aac agg aac aag aac aag aca gca ggc tcc cag       960
Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln
305                 310                 315                 320

gaa gcc ccc aac ctc cct cgt aag cgc aga ttc tca cct cac act gtc      1008
Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val
                325                 330                 335

ctt ggc cgg ttc ttc gag agc tgg gga aca agg gtg gcc tca tgg cca      1056
Leu Gly Arg Phe Phe Glu Ser Trp Gly Thr Arg Val Ala Ser Trp Pro
        340                 345                 350

ctc act gtc ttg gca ctg tcc ttc ata gtt gtg ata gcc ttg tca gta      1104
Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val
        355                 360                 365

ggc ctg acc ttt ata gaa ctc acc aca gac cct gtg gaa ctg tgg tcg      1152
Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
        370                 375                 380

gcc cct aaa agc caa gcc cgg aaa gaa aag gct ttc cat gac gag cat      1200
Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His
385                 390                 395                 400

ttt ggc ccc ttc ttc cga acc aac cag att ttt gtg aca gct aag aac      1248
Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn
                405                 410                 415
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agg tcc agc tac aag tac gac tcc ctg ctg cta ggg ccc aag aac ttc      1296
Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
            420             425             430

agt ggg atc cta tcc ctg gac ttg ctg cag gag ctg ttg gag cta cag      1344
Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln
            435             440             445

gag aga ctt cga cac ctg caa gtg tgg tcc cat gag gca cag cgc aac      1392
Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn
        450             455             460

atc tcc ctc cag gac atc tgc tat gct ccc ctc aac ccg cat aac acc      1440
Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr
465             470             475             480

agc ctc act gac tgc tgt gtc aac agc ctc ctt caa tac ttc cag aac      1488
Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                485             490             495

aac cac aca ctc ctg ctg ctc aca gcc aat cag act ctg aat ggc cag      1536
Asn His Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
            500             505             510

acc tcc ctg gtg gac tgg aag gac cat ttc ctc tac tgt gcc aat gcc      1584
Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
            515             520             525

cct ctc acg tac aaa gat ggc aca gcc ctg gcc ctg agc tgc ata gct      1632
Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala
        530             535             540

gac tac ggg gca cct gtc ttc ccc ttc ctt gct gtt ggg ggc tac caa      1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545             550             555             560

ggg acg gac tac tcg gag gca gaa gcc ctg atc ata acc ttc tct atc      1728
Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile
                565             570             575

aat aac tac ccc gct gat gat ccc cgc atg gcc cac gcc aag ctc tgg      1776
Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp
            580             585             590

gag gag gct ttc ttg aag gaa atg caa tcc ttc cag aga agc aca gct      1824
Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala
            595             600             605

gac aag ttc cag att gcg ttc tca gct gag cgt tct ctg gag gac gag      1872
Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
            610             615             620

atc aat cgc act acc atc cag gac ctg cct gtc ttt gcc atc agc tac      1920
Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr
625             630             635             640

ctt atc gtc ttc ctg tac atc tcc ctg gcc ctg ggc agc tac tcc aga      1968
Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
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                  645                    650                     655

· tgg agc cga gtt gcg gtg gat tcc aag gct act ctg ggc cta ggt ggg      2016
  Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
              660                 665               670

  gtg gct gtt gtg ctg gga gca gtc gtc gct gcc atg ggc ttc tac tcc      2064
  Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser
              675                 680               685

  tac ctg ggt gtc ccc tcc tct ctg gtc atc att caa gtg gta cct ttc      2112
  Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
              690                 695               700

  ctg gtg ctg gct gtg gga gct gac aac atc ttc atc ttt gtt ctt gag      2160
  Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
  705                 710                 715               720

  tac cag agg ctg cct agg atg ccc ggg gag cag cga gag gct cac att      2208
  Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
                  725                 730               735

  ggc cgc acc ctg ggt agt gtg gcc ccc agc atg ctg ctg tgc agc ctc      2256
  Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
              740                 745               750

  tct gag gcc atc tgc ttc ttt cta ggg gcc ctg acc tcc atg cca gct      2304
  Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala
              755                 760               765

  gtg agg acc ttt gcc ttg acc tct ggc tta gca atc atc ttt gac ttc      2352
  Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe
  770                 775                 780

  ctg ctc cag atg aca gcc ttt gtg gcc ctg ctc tcc ctg gat agc aag      2400
  Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
  785                 790                 795               800

  agg cag gag gcc tct cgc ccc gac gtc gtg tgc tgc ttt tca agc cga      2448
  Arg Gln Glu Ala Ser Arg Pro Asp Val Val Cys Cys Phe Ser Ser Arg
                  805                 810               815

  aat ctg ccc cca ccg aaa caa aaa gaa ggc ctc tta ctt tgc ttc ttc      2496
  Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe
                  820                 825               830

  cgc aag ata tac act ccc ttc ctg ctg cac aga ttc atc cgc cct gtt      2544
  Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
              835                 840               845

  gtg ctg ctg ctc ttt ctg gtc ctg ttt gga gca aac ctc tac tta atg      2592
  Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met
              850                 855               860

  tgc aac atc agc gtg ggg ctg gac cag gat ctg gct ctg ccc aag gat      2640
  Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp
  865                 870                 875               880

  tcc tac ctg ata gac tac ttc ctc ttt ctg aac cgg tac ttg gaa gtg      2688
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
            885                 890                 895

ggg cct cca gtg tac ttt gac acc acc tca ggc tac aac ttt tcc acc   2736
Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr
            900                 905                 910

gag gca ggc atg aac gcc att tgc tct agt gca ggc tgt gag agc ttc   2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe
            915                 920                 925

tcc cta acc cag aaa atc cag tat gcc agt gaa ttc cct aat cag tct   2832
Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser
        930                 935                 940

tat gtg gct att gct gca tcc tcc tgg gta gat gac ttc atc gac tgg   2880
Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945             950                 955                 960

ctg acc cca tcc tcc tcc tgc tgc cgc att tat acc cgt ggc ccc cat   2928
Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His
            965                 970                 975

aaa gat gag ttc tgt ccc tca acg gat act tcc ttc aac tgt ctc aaa   2976
Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
            980                 985                 990

aac tgc atg aac cgc act ctg ggt  ccc gtg aga ccc aca  aca gaa cag   3024
Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr  Thr Glu Gln
        995                 1000                1005

ttt cat  aag tac ctg ccc tgg  ttc ctg aat gat acg  ccc aac atc   3069
Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr  Pro Asn Ile
    1010                1015                1020

aga tgt  cct aaa ggg ggc cta  gca gcg tat aga acc  tct gtg aat   3114
Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr  Ser Val Asn
    1025                1030                1035

ttg agc  tca gat ggc cag att  ata gcc tcc cag ttc  atg gcc tac   3159
Leu Ser Ser Asp Gly Gln Ile Ile Ala Ser Gln Phe Met Ala Tyr
    1040                1045                1050

cac aag  ccc tta cgg aac tca  cag gac ttt aca gaa  gct ctc cgg   3204
His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu  Ala Leu Arg
    1055                1060                1065

gca tcc  cgg ttg cta gca gcc  aac atc aca gct gaa  cta cgg aag   3249
Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Glu  Leu Arg Lys
    1070                1075                1080

gtg cct  ggg aca gat ccc aac  ttt gag gtc ttc cct  tac acg atc   3294
Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro  Tyr Thr Ile
    1085                1090                1095

tcc aat  gtg ttc tac cag caa  tac ctg acg gtt ctc  cct gag gga   3339
Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu  Pro Glu Gly
    1100                1105                1110
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atc ttc act ctt gct ctc tgc  ttc gtg ccc acc ttt  gtg gtc tgc        3384
Ile Phe Thr Leu Ala Leu Cys  Phe Val Pro Thr Phe  Val Val Cys
    1115                1120                1125

tac ctc cta ctg ggc ctg gac  ata cgc tca ggc atc  ctc aac ctg        3429
Tyr Leu Leu Leu Gly Leu Asp  Ile Arg Ser Gly Ile  Leu Asn Leu
    1130                1135                1140

ctc tcc atc att atg atc ctc  gtg gac acc atc ggc  ctc atg gct        3474
Leu Ser Ile Ile Met Ile Leu  Val Asp Thr Ile Gly  Leu Met Ala
    1145                1150                1155

gtg tgg ggt atc agc tac aat  gct gtg tcc ctc atc  aac ctt gtc        3519
Val Trp Gly Ile Ser Tyr Asn  Ala Val Ser Leu Ile  Asn Leu Val
    1160                1165                1170

acg gca gtg ggc atg tct gtg  gag ttc gtg tcc cac  att acc cgg        3564
Thr Ala Val Gly Met Ser Val  Glu Phe Val Ser His  Ile Thr Arg
    1175                1180                1185

tcc ttt gct gta agc acc aag  cct acc cgg ctg gag  aga gcc aaa        3609
Ser Phe Ala Val Ser Thr Lys  Pro Thr Arg Leu Glu  Arg Ala Lys
    1190                1195                1200

gat gct act atc ttc atg ggc  agt gcg gtg ttt gct  gga gtg gcc        3654
Asp Ala Thr Ile Phe Met Gly  Ser Ala Val Phe Ala  Gly Val Ala
    1205                1210                1215

atg acc aac ttc ccg ggc atc  ctc atc ctg ggc ttt  gct cag gcc        3699
Met Thr Asn Phe Pro Gly Ile  Leu Ile Leu Gly Phe  Ala Gln Ala
    1220                1225                1230

cag ctt atc cag att ttc ttc  ttc cgc ctc aac ctc  ctg atc acc        3744
Gln Leu Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
    1235                1240                1245

ttg ctg ggt ctg cta cac ggc  ctg gtc ttc ctg ccc  gtt gtc ctc        3789
Leu Leu Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
    1250                1255                1260

agc tat ctg ggg cca gat gtt  aac caa gct ctg gta  ctg gag gag        3834
Ser Tyr Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Leu Glu Glu
    1265                1270                1275

aaa cta gcc act gag gca gcc  atg gtc tca gag cct  tct tgc cca        3879
Lys Leu Ala Thr Glu Ala Ala  Met Val Ser Glu Pro  Ser Cys Pro
    1280                1285                1290

cag tac ccc ttc ccg gct gat  gca aac acc agt gac  tat gtt aac        3924
Gln Tyr Pro Phe Pro Ala Asp  Ala Asn Thr Ser Asp  Tyr Val Asn
    1295                1300                1305

tac ggc ttt aat cca gaa ttt  atc cct gaa att aat  gct gct agc        3969
Tyr Gly Phe Asn Pro Glu Phe  Ile Pro Glu Ile Asn  Ala Ala Ser
    1310                1315                1320

agc tct ctg ccc aaa agt gac  caa aag ttc taa                         4002
Ser Ser Leu Pro Lys Ser Asp  Gln Lys Phe
    1325                1330
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Ser Ala Gln Gly Glu Leu Tyr Thr Pro Thr His Lys Ala Gly Phe Cys
            20   25   30

Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
            35   40   45

Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His
    50   55   60

Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu
65   70   75   80

Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu
                85   90   95

Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100   105   110

Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr
            115   120   125

Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln
    130   135   140

Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145   150   155   160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr

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    Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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    Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
            210   215   220

    Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile
    225   230   235   240

    Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys
                245   250   255

    Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Ala Leu
                260   265   270

    Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
                275   280   285

    Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr
            290   295   300

    Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln
    305   310   315   320

    Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val
                325   330   335

    Leu Gly Arg Phe Phe Glu Ser Trp Gly Thr Arg Val Ala Ser Trp Pro
                340   345   350

    Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val
                355   ·   360   365

    Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
        370   375   380

    Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His
    385   390   395   400

    Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn
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Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
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Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln
        435             440             445

Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn
    450             455             460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr
465             470             475             480

Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
            485             490             495

Asn His Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
        500             505             510

Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515             520             525

Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala
    530             535             540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545             550             555             560

Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile
            565             570             575

Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp
            580             585             590

Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala
        595             600             605

Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
    610             615             620

Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr
625             630             635             640

Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
            645             650             655
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Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
        660   665   670

Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser
        675   680   685

Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
    690   695   700

Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705   710   715   720

Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
            725   730   735

Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
        740   745   750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala
        755   760   765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe
    770   775   780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785   790   795   800

Arg Gln Glu Ala Ser Arg Pro Asp Val Val Cys Cys Phe Ser Ser Arg
            805   810   815

Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe
            820   825   830

Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
        835   840   845

Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met
    850   855   860

Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp
865   870   875   880

Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
            885   890   895

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Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr
        900             905             910

Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe
        915             920             925

Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser
        930             935             940

Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945             950             955             960

Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His
                965             970             975

Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
            980             985             990

Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Thr Glu Gln
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Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr Pro Asn Ile
    1010            1015            1020

Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn
    1025            1030            1035

Leu Ser Ser Asp Gly Gln Ile Ile Ala Ser Gln Phe Met Ala Tyr
    1040            1045            1050

His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg
    1055            1060            1065

Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Glu Leu Arg Lys
    1070            1075            1080

Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile
    1085            1090            1095

Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly
    1100            1105            1110

Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys
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            1115                 1120                 1125

   Tyr Leu   Leu Leu Gly Leu Asp   Ile Arg Ser Gly Ile   Leu Asn Leu
       1130              1135              1140

   Leu Ser   Ile Ile Met Ile Leu   Val Asp Thr Ile Gly   Leu Met Ala
       1145              1150              1155

   Val Trp   Gly Ile Ser Tyr Asn   Ala Val Ser Leu Ile   Asn Leu Val
       1160              1165              1170

   Thr Ala   Val Gly Met Ser Val   Glu Phe Val Ser His   Ile Thr Arg
       1175              1180              1185

   Ser Phe   Ala Val Ser Thr Lys   Pro Thr Arg Leu Glu   Arg Ala Lys
       1190              1195              1200

   Asp Ala   Thr Ile Phe Met Gly   Ser Ala Val Phe Ala   Gly Val Ala
       1205              1210              1215

   Met Thr   Asn Phe Pro Gly Ile   Leu Ile Leu Gly Phe   Ala Gln Ala
       1220              1225              1230

   Gln Leu   Ile Gln Ile Phe Phe   Phe Arg Leu Asn Leu   Leu Ile Thr
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   Leu Leu   Gly Leu Leu His Gly   Leu Val Phe Leu Pro   Val Val Leu
       1250              1255              1260

   Ser Tyr   Leu Gly Pro Asp Val   Asn Gln Ala Leu Val   Leu Glu Glu
       1265              1270              1275

   Lys Leu   Ala Thr Glu Ala Ala   Met Val Ser Glu Pro   Ser Cys Pro
       1280              1285              1290

   Gln Tyr   Pro Phe Pro Ala Asp   Ala Asn Thr Ser Asp   Tyr Val Asn
       1295              1300              1305

   Tyr Gly   Phe Asn Pro Glu Phe   Ile Pro Glu Ile Asn   Ala Ala Ser
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   Ser Ser   Leu Pro Lys Ser Asp   Gln Lys Phe
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wsntayytna thgaytaytt yytnttyytn aaymgntayy tngargtngg nccnccngtn    2700

tayttygaya cnacnwsngg ntayaaytty wsnacngarg cnggnatgaa ygcnathtgy    2760

wsnwsngcng gntgygarws nttywsnytn acncaraara thcartaygc nwsngartty    2820

ccnaaycarw sntaygtngc nathgcngcn wsnwsntggg tngaygaytt yathgaytgg    2880

ytnacnccnw snwsnwsntg ytgymgnath tayacnmgng ncccncayaa rgaygartty    2940

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ytnmgnaarg tnccnggnac ngayccnaay ttygargtnt tyccntayac nathwsnaay      3300

gtnttytayc arcartayyt nacngtnytn ccngarggna thttyacnyt ngcnytntgy      3360

ttygtnccna cnttygtngt ntgytayytn ytnytnggny tngayathmg nwsnggnath      3420

ytnaayytny tnwsnathat hatgathytn gtngayacna thggnytnat ggcngtntgg      3480

ggnathwsnt ayaaygcngt nwsnytnath aayytngtna cngcngtngg natgwsngtn      3540

garttygtnw sncayathac nmgnwsntty gcngtnwsna cnaarccnac nmgnytngar      3600

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aayttyccng gnathytnat hytnggntty gcncargcnc arytnathca rathttytty      3720

ttymgnytna ayytnytnat hacnytnytn ggnytnytnc ayggnytngt nttyytnccn      3780

gtngtnytnw sntayytngg nccngaygtn aaycargcny tngtnytnga rgaraarytn      3840

gcnacngarg cngcnatggt nwsngarccn wsntgyccnc artayccntt yccngcngay      3900

gcnaayacnw sngaytaygt naaytayggn ttyaayccng arttyathcc ngarathaay      3960

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Asp Tyr Lys Asp Asp Asp Asp Lys
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Tyr Gln Arg Leu
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Glu Gln Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn
1   5   10   15

Ile Arg Cys

<210>   40

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Glu Ala Phe Tyr Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys
1   5   10   15

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<213>  Artificial Sequence


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Gly Gln Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys
1               5                   10                  15


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<213>  Artificial Sequence


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Cys Ala Asn Ala Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu
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Ser

<210>  43

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                                                                      Met
                                                                       1

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Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu Arg
            5   10   15

ttg gcc cag agt gag cct tac aca acc atc cac cag cct ggc tac tgc   155
Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr Cys
        20   25   30

gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc ctc   203
Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser Leu
    35   40   45

atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc aag   251
Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg Lys
50   55   60   65

atc aca ggt gat cac ctg atc cta tta cag aag atc tgc ccc cgc ctc   299
Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg Leu
                70   75   80

tac acc ggc ccc aac acc caa gcc tgc tgc tcc gcc aag cag ctg gta   347
Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu Val
            85   90   95

tca ctg gaa gcg agt ctg tcg atc acc aag gcc ctc ctc acc cgc tgc   395
Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg Cys
        100   105   110

cca gcc tgc tct gac aat ttt gtg aac ctg cac tgc cac aac acg tgc   443
Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr Cys
    115   120   125

agc ccc aat cag agc ctc ttc atc aat gtg acc cgc gtg gcc cag cta   491
Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln Leu
130   135   140   145

ggg gct gga caa ctc cca gct gtg gtg gcc tat gag gcc ttc tac cag   539
Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr Gln
                150   155   160

cat agc ttt gcc gag cag agc tat gac tcc tgc agc cgt gtg cgc gtc   587
His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg Val
            165   170   175

cct gca gct gcc acg ctg gct gtg ggc acc atg tgt ggc gtg tat ggc   635
Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr Gly
        180   185   190

tct gcc ctt tgc aat gcc cag cgc tgg ctc aac ttc cag gga gac aca   683

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        Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp Thr
            195             200             205

        ggc aat ggt ctg gcc cca ctg gac atc acc ttc cac ctc ttg gag cct    731
        Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu Pro
        210             215             220             225

        ggc cag gcc gtg ggg agt ggg att cag cct ctg aat gag ggg gtt gca    779
        Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val Ala
                        230             235             240

        cgt tgc aat gag tcc caa ggt gac gac gtg gcg acc tgc tcc tgc caa    827
        Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys Gln
                        245             250             255

        gac tgt gct gca tcc tgt cct gcc ata gcc cgc ccc cag gcc ctc gac    875
        Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu Asp
                260             265             270

        tcc acc ttc tac ctg ggc cag atg ccg ggc agt ctg gtc ctc atc atc    923
        Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile Ile
            275             280             285

        atc ctc tgc tct gtc ttc gct gtg gtc acc atc ctg ctt gtg gga ttc    971
        Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly Phe
        290             295             300             305

        cgt gtg gcc ccc gcc agg gac aaa agc aag atg gtg gac ccc aag aag    1019
        Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys Lys
                        310             315             320

        ggc acc agc ctc tct gac aag ctc agc ttc tcc acc cac acc ctc ctt    1067
        Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu Leu
                325             330             335

        ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct ctg    1115
        Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro Leu
                340             345             350

        acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg ggc    1163
        Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala Gly
            355             360             365

        ctg gtc ttt aca gaa ctc act acg gac ccc gtg gag ctg tgg tcg gcc    1211
        Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser Ala
        370             375             380             385

        ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat ttc    1259
        Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His Phe
                        390             395             400

        ggc ccc ttc ttc cga acc aac cag gtg atc ctg acg gct cct aac cgg    1307
        Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn Arg
                405             410             415

        tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc agc    1355
        Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe Ser
            420             425             430
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gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag gag    1403
Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln Glu
    435             440             445

agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac atc    1451
Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn Ile
450             455             460             465

tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc agt    1499
Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr Ser
            470             475             480

ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac aac    1547
Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn Asn
            485             490             495

cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag acc    1595
Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln Thr
        500             505             510

tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc ccg    1643
Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala Pro
    515             520             525

ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct gac    1691
Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala Asp
530             535             540             545

tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa gga    1739
Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys Gly
            550             555             560

aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc aac    1787
Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu Asn
            565             570             575

aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg gag    1835
Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp Glu
        580             585             590 .

gag gcc ttc tta gag gaa atg cga gcc ttc cag cgt cgg atg gct ggc    1883
Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala Gly
    595             600             605

atg ttc cag gtc acg ttc atg gct gag cgc tct ctg gaa gac gag atc    1931
Met Phe Gln Val Thr Phe Met Ala Glu Arg Ser Leu Glu Asp Glu Ile
610             615             620             625

aat cgc acc aca gct gaa gac ctg ccc atc ttt gcc acc agc tac att    1979
Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr Ile
            630             635             640

gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc tgg    2027
Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser Trp
            645             650             655

agc cga gtg atg gtg gac tcc aag gcc acg ctg ggc ctc ggc ggg gtg    2075
Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly Val
            660             665             670
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gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc tac        2123
Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser Tyr
    675             680             685

ttg ggt atc cgc tcc tcc ctg gtc atc ctg caa gtg gtt cct ttc ctg        2171
Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe Leu
690             695             700             705

gtg ctg tcc gtg ggg gct gat aac atc ttc atc ttt gtt ctc gag tac        2219
Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu Tyr
                710             715             720

cag agg ctg ccc cgg agg cct ggg gag cca cga gag gtc cac att ggg        2267
Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile Gly
            725             730             735

cga gcc cta ggc agg gtg gct ccc agc atg ctg ttg tgc agc ctc tct        2315
Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu Ser
        740             745             750

gag gcc atc tgc ttc ttc cta ggg gcc ctg acc ccc atg cca gct gtg        2363
Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala Val
    755             760             765

cgg acc ttt gcc ctg acc tct ggc ctt gca gtg atc ctt gac ttc ctc        2411
Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe Leu
770             775             780             785

ctg cag atg tca gcc ttt gtg gcc ctg ctc tcc ctg gac agc aag agg        2459
Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys Arg
            790             795             800

cag gag gcc tcc cgg ttg gac gtc tgc tgc tgt gtc aag ccc cag gag        2507
Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln Glu
        805             810             815

ctg ccc ccg cct ggc cag gga gag ggg ctc ctg ctt ggc ttc ttc caa        2555
Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe Gln
        820             825             830

aag gct tat gcc ccc ttc ctg ctg cac tgg atc act cga ggt gtt gtg        2603
Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val Val
    835             840             845

ctg ctg ctg ttt ctc gcc ctg ttc gga gtg agc ctc tac tcc atg tgc        2651
Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met Cys
850             855             860             865

cac atc agc gtg gga ctg gac cag gag ctg gcc ctg ccc aag gac tcg        2699
His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp Ser
                870             875             880

tac ctg ctt gac tat ttc ctc ttt ctg aac cgc tac ttc gag gtg ggg        2747
Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val Gly
            885             890             895

gcc ccg gtg tac ttt gtt acc acc ttg ggc tac aac ttc tcc agc gag        2795
Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser Glu
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                   900                      905                      910

       gct ggg atg aat gcc atc tgc tcc agt gca ggc tgc aac aac ttc tcc    2843
       Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe Ser
           915                  920                  925

       ttc acc cag aag atc cag tat gcc aca gag ttc cct gag cag tct tac    2891
       Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser Tyr
       930                  935                  940                  945

       ctg gcc atc cct gcc tcc tcc tgg gtg gat gac ttc att gac tgg ctg    2939
       Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp Leu
                           950                  955                  960

       acc ccg tcc tcc tgc tgc cgc ctt tat ata tct ggc ccc aat aag gac    2987
       Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys Asp
                       965                  970                  975

       aag ttc tgc ccc tcg acc gtc aac tct ctg aac tgc cta aag aac tgc    3035
       Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn Cys
               980                  985                  990

       atg agc atc acg atg ggc tct  gtg agg ccc tcg gtg  gag cag ttc cat  3083
       Met Ser Ile Thr Met Gly Ser  Val Arg Pro Ser Val  Glu Gln Phe His
           995                  1000                 1005

       aag  tat ctt ccc tgg ttc  ctg aac gac cgg ccc  aac atc aaa tgt     3128
       Lys  Tyr Leu Pro Trp Phe  Leu Asn Asp Arg Pro  Asn Ile Lys Cys
       1010                 1015                 1020

       ccc  aaa ggc ggc ctg gca  gca tac agc acc tct  gtg aac ttg act     3173
       Pro  Lys Gly Gly Leu Ala  Ala Tyr Ser Thr Ser  Val Asn Leu Thr
       1025                 1030                 1035

       tca  gat ggc cag gtt tta  gac aca gtt gcc att  ctg tca ccc agg     3218
       Ser  Asp Gly Gln Val Leu  Asp Thr Val Ala Ile  Leu Ser Pro Arg
       1040                 1045                 1050

       ctg  gag tac agt ggc aca  atc tcg gct cac tgc  aac ctc tac ctc     3263
       Leu  Glu Tyr Ser Gly Thr  Ile Ser Ala His Cys  Asn Leu Tyr Leu
       1055                 1060                 1065

       ctg  gat tca gcc tcc agg  ttc atg gcc tat cac  aag ccc ctg aaa     3308
       Leu  Asp Ser Ala Ser Arg  Phe Met Ala Tyr His  Lys Pro Leu Lys
       1070                 1075                 1080

       aac  tca cag gat tac aca  gaa gct ctg cgg gca  gct cga gag ctg     3353
       Asn  Ser Gln Asp Tyr Thr  Glu Ala Leu Arg Ala  Ala Arg Glu Leu
       1085                 1090                 1095

       gca  gcc aac atc act gct  gac ctg cgg aaa gtg  cct gga aca gac     3398
       Ala  Ala Asn Ile Thr Ala  Asp Leu Arg Lys Val  Pro Gly Thr Asp
       1100                 1105                 1110

       ccg  gct ttt gag gtc ttc  ccc tac acg atc acc  aat gtg ttt tat     3443
       Pro  Ala Phe Glu Val Phe  Pro Tyr Thr Ile Thr  Asn Val Phe Tyr
       1115                 1120                 1125

       gag  cag tac ctg acc atc  ctc cct gag ggg ctc  ttc atg ctc agc     3488
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Glu  Gln  Tyr  Leu  Thr  Ile   Leu  Pro  Glu  Gly  Leu   Phe  Met  Leu  Ser
1130                1135                      1140

ctc  tgc  ctt  gtg  ccc  acc   ttc  gct  gtc  tcc  tgc   ctc  ctg  ctg  ggc    3533
Leu  Cys  Leu  Val  Pro  Thr   Phe  Ala  Val  Ser  Cys   Leu  Leu  Leu  Gly
1145                1150                      1155

ctg  gac  ctg  cgc  tcc  ggc   ctc  ctc  aac  ctg  ctc   tcc  att  gtc  atg    3578
Leu  Asp  Leu  Arg  Ser  Gly   Leu  Leu  Asn  Leu  Leu   Ser  Ile  Val  Met
1160                1165                      1170

atc  ctc  gtg  gac  act  gtc   ggc  ttc  atg  gcc  ctg   tgg  ggc  atc  agt    3623
Ile  Leu  Val  Asp  Thr  Val   Gly  Phe  Met  Ala  Leu   Trp  Gly  Ile  Ser
1175                1180                      1185

tac  aat  gct  gtg  tcc  ctc   atc  aac  ctg  gtc  tcg   gcg  gtg  ggc  atg    3668
Tyr  Asn  Ala  Val  Ser  Leu   Ile  Asn  Leu  Val  Ser   Ala  Val  Gly  Met
1190                1195                      1200

tct  gtg  gag  ttt  gtg  tcc   cac  att  acc  cgc  tcc   ttt  gcc  atc  agc    3713
Ser  Val  Glu  Phe  Val  Ser   His  Ile  Thr  Arg  Ser   Phe  Ala  Ile  Ser
1205                1210                      1215

acc  aag  ccc  acc  tgg  ctg   gag  agg  gcc  aaa  gag   gcc  acc  atc  tct    3758
Thr  Lys  Pro  Thr  Trp  Leu   Glu  Arg  Ala  Lys  Glu   Ala  Thr  Ile  Ser
1220                1225                      1230

atg  gga  agt  gcg  gtg  ttt   gca  ggt  gtg  gcc  atg   acc  aac  ctg  cct    3803
Met  Gly  Ser  Ala  Val  Phe   Ala  Gly  Val  Ala  Met   Thr  Asn  Leu  Pro
1235                1240                      1245

ggc  atc  ctt  gtc  ctg  ggc   ctc  gcc  aag  gcc  cag   ctc  att  cag  atc    3848
Gly  Ile  Leu  Val  Leu  Gly   Leu  Ala  Lys  Ala  Gln   Leu  Ile  Gln  Ile
1250                1255                      1260

ttc  ttc  ttc  cgc  ctc  aac   ctc  ctg  atc  act  ctg   ctg  ggc  ctg  ctg    3893
Phe  Phe  Phe  Arg  Leu  Asn   Leu  Leu  Ile  Thr  Leu   Leu  Gly  Leu  Leu
1265                1270                      1275

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His  Gly  Leu  Val  Phe  Leu   Pro  Val  Ile  Leu  Ser   Tyr  Val  Gly  Pro
1280                1285                      1290

gac  gtt  aac  ccg  gct  ctg   gca  ctg  gag  cag  aag   cgg  gct  gag  gag    3983
Asp  Val  Asn  Pro  Ala  Leu   Ala  Leu  Glu  Gln  Lys   Arg  Ala  Glu  Glu
1295                1300                      1305

gcg  gtg  gca  gca  gtc  atg   gtg  gcc  tct  tgc  cca   aat  cac  ccc  tcc    4028
Ala  Val  Ala  Ala  Val  Met   Val  Ala  Ser  Cys  Pro   Asn  His  Pro  Ser
1310                1315                      1320

cga  gtc  tcc  aca  gct  gac   aac  atc  tat  gtc  aac   cac  agc  ttt  gaa    4073
Arg  Val  Ser  Thr  Ala  Asp   Asn  Ile  Tyr  Val  Asn   His  Ser  Phe  Glu
1325                1330                      1335

ggt  tct  atc  aaa  ggt  gct   ggt  gcc  atc  agc  aac   ttc  ttg  ccc  aac    4118
Gly  Ser  Ile  Lys  Gly  Ala   Gly  Ala  Ile  Ser  Asn   Phe  Leu  Pro  Asn
1340                1345                      1350
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Asn  Gly Arg Gln Phe
1355

gccctgaacc aaagggttat ggggatcttc cttgtgactg ccccttgaca cacgccctcc   4226

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caagtcacct aggccttggg cagtgcctcc tcctgggcct gggtctggaa gttggcagga   4406

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caacttttcc cttctcagtt cctctctcga aagtcttaat tctgtgtcag taagtcttta   4526

acacgtagca gtgtccctga gaacacagac aatgaccact accctgggtg tgatatcaca   4586

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gagcttttgg acagctctcc agaagcatga actgcagtgg agatgaagat cctggctctg   5006

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<211> 1359

<212> PRT

<213> Homo sapiens

<400> 44

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            20                  25                  30

Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
            35                  40                  45
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Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
    50              55          60

Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
65              70          75              80

Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
                85          90              95

Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100         105         110

Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
        115         120         125

Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
    130         135         140

Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
    145         150         155         160

Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
            165         170         175

Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
        180         185         190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
    195         200         205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
    210         215         220

Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
225         230         235         240

Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
            245         250         255

Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
        260         265         270

Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
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                275                 280                 285


     Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
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     Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
     305                 310                 315                 320


     Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
                 325                 330                 335


     Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
                 340                 345                 350


     Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
                 355                 360                 365


     Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
         370                 375                 380


     Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
     385                 390                 395                 400


     Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
                 405                 410                 415


     Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
                 420                 425                 430


     Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
             435                 440                 445


     Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
         450                 455                 460


     Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
     465                 470                 475                 480


     Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                 485                 490                 495


     Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
                 500                 505                 510
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Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515             520             525

Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
    530             535             540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
545             550             555             560

Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
            565             570             575

Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
        580             585             590

Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
    595             600             605

Gly Met Phe Gln Val Thr Phe Met Ala Glu Arg Ser Leu Glu Asp Glu
    610             615             620

Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625             630             635             640

Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
            645             650             655

Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
            660             665             670

Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
        675             680             685

Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
    690             695             700

Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705             710             715             720

Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
            725             730             735

Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740             745             750
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Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
     755   760   765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
    770   775   780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785   790   795   800

Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Val Lys Pro Gln
          805   810   815

Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
       820   825   830

Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
     835   840   845

Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
    850   855   860

Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865   870   875   880

Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
          885   890   895

Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
       900   905   910

Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
       915   920   925

Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
    930   935   940

Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945   950   955   960

Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys
          965   970   975

Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
          980   985   990

Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe
        995   1000   1005

His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys
    1010   1015   1020

Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu
    1025   1030   1035

Thr Ser Asp Gly Gln Val Leu Asp Thr Val Ala Ile Leu Ser Pro
    1040   1045   1050

Arg Leu Glu Tyr Ser Gly Thr Ile Ser Ala His Cys Asn Leu Tyr
    1055   1060   1065

Leu Leu Asp Ser Ala Ser Arg Phe Met Ala Tyr His Lys Pro Leu
    1070   1075   1080

Lys Asn Ser Gln Asp Tyr Thr Glu Ala Leu Arg Ala Ala Arg Glu
    1085   1090   1095

Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys Val Pro Gly Thr
    1100   1105   1110

Asp Pro Ala Phe Glu Val Phe Pro Tyr Thr Ile Thr Asn Val Phe
    1115   1120   1125

Tyr Glu Gln Tyr Leu Thr Ile Leu Pro Glu Gly Leu Phe Met Leu
    1130   1135   1140

Ser Leu Cys Leu Val Pro Thr Phe Ala Val Ser Cys Leu Leu Leu
    1145   1150   1155

Gly Leu Asp Leu Arg Ser Gly Leu Leu Asn Leu Leu Ser Ile Val
    1160   1165   1170

Met Ile Leu Val Asp Thr Val Gly Phe Met Ala Leu Trp Gly Ile
    1175   1180   1185

Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val Ser Ala Val Gly
    1190   1195   1200

Met Ser Val Glu Phe Val Ser His Ile Thr Arg Ser Phe Ala Ile

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        1205                  1210                  1215


  Ser Thr Lys Pro Thr Trp Leu Glu Arg Ala Lys Glu Ala Thr Ile
      1220                  1225                  1230


  Ser Met Gly Ser Ala Val Phe Ala Gly Val Ala Met Thr Asn Leu
      1235                  1240                  1245


  Pro Gly Ile Leu Val Leu Gly Leu Ala Lys Ala Gln Leu Ile Gln
      1250                  1255                  1260


  Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr Leu Leu Gly Leu
      1265                  1270                  1275


  Leu His Gly Leu Val Phe Leu Pro Val Ile Leu Ser Tyr Val Gly
      1280                  1285                  1290


  Pro Asp Val Asn Pro Ala Leu Ala Leu Glu Gln Lys Arg Ala Glu
      1295                  1300                  1305


  Glu Ala Val Ala Ala Val Met Val Ala Ser Cys Pro Asn His Pro
      1310                  1315                  1320


  Ser Arg Val Ser Thr Ala Asp Asn Ile Tyr Val Asn His Ser Phe
      1325                  1330                  1335


  Glu Gly Ser Ile Lys Gly Ala Gly Ala Ile Ser Asn Phe Leu Pro
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  Asn Asn Gly Arg Gln Phe
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<211>  4471

<212>  DNA

<213>  Mus musculus


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caagcagctg gtgtcattag acagtagcct gtctatcacc aaggccctcc ttacacgctg    360

cccggcatgc tctgaaaatt ttgtgagcat acactgtcat aatacctgca gccctgacca    420

gagcctcttc atcaatgtta ctcgcgtggt tcagcgggac cctggacagc ttcctgctgt    480

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<213>  Artificial sequence


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<220>

<223>  primer

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<211>  25

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<213>  Artificial sequence


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<212> DNA

<213> Homo sapiens

<220>

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<222> (1)..(3996)

<223> n is g or a or t or c

<400> 51

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aaraayccng arytnwsngg nwsnytnatg acnytnwsna aygtnwsntg yytnwsnaay     180

acnccngcnm gnaarathac nggngaycay ytnathytny tncaraarat htgyccnmgn     240

ytntayacng gnccnaayac ncargcntgy tgywsngcna arcarytngt nwsnytngar     300

gcnwsnytnw snathacnaa rgcnytnytn acnmgntgyc cngcntgyws ngayaaytty     360

gtnaayytnc aytgycayaa yacntgywsn ccnaaycarw snytnttyat haaygtnacn     420

mgngtngcnc arytnggngc nggncarytn ccngcngtng tngcntayga rgcnttytay     480

carcaywsnt tygcngarca rwsntaygay wsntgywsnm gngtnmgngt nccngcngcn     540

gcnacnytng cngtnggnac natgtgyggn gtntayggnw sngcnytntg yaaygcncar     600

mgntggytna ayttycargg ngayacnggn aayggnytng cnccnytnga yathacntty     660

cayytnytng arccnggnca rgcngtnggn wsnggnathc arccnytnaa ygarggngtn     720

gcnmgntgya aygarwsnca rggngaygay gtngcnacnt gywsntgyca rgaytgygcn     780

gcnwsntgyc cngcnathgc nmgnccncar gcnytngayw snacnttyta yytnggncar     840

atgccnggnw snytngtnyt nathathath ytntgywsng tnttygcngt ngtnacnath     900

ytnytngtng gnttymgngt ngcnccngcn mgngayaarw snaaratggt ngayccnaar     960

aarggnacnw snytnwsnga yaarytnwsn ttywsnacnc ayacnytnyt nggncartty    1020

ttycarggnt ggggnacntg ggtngcnwsn tggccnytna cnathytngt nytnwsngtn    1080

athccngtng tngcnytngc ngcnggnytn gtnttyacng arytnacnac ngayccngtn    1140

garytntggw sngcnccnaa ywsncargcn mgnwsngara argcnttyca ygaycarcay    1200
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ttyggnccnt  tyttymgnac  naaycargtn  athytnacng  cnccnaaymg  nwsnwsntay   1260

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ytnytngary  tnytngaryt  ncargarmgn  ytnmgncayy  tncargtntg  gwsnccngar   1380

gcncarmgna  ayathwsnyt  ncargayath  tgytaygcnc  cnytnaaycc  ngayaayacn   1440

wsnytntayg  aytgytgyat  haaywsnytn  ytncartayt  tycaraayaa  ymgnacnytn   1500

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mgngcnttyc  armgnmgnat  ggcnggnatg  ttycargtna  cnttyacngc  ngarmgnwsn   1860

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wsnwsngcng  gntgyaayaa  yttywsntty  acncaraara  thcartaygc  nacngartty   2820

ccngarcarw  sntayytngc  nathccngcn  wsnwsntggg  tngaygaytt  yathgaytgg   2880

ytnacnccnw  snwsntgytg  ymgnytntay  athwsnggnc  naayaarga  yaarttytgy   2940
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gtnmgnccnw  sngtngarca  rttycayaar  tayytnccnt  ggttyytnaa  ygaymgnccn       3060

aayathaart  gyccnaargg  nggnytngcn  gcntaywsna  cnwsngtnaa  yytnacnwsn       3120

gayggncarg  tnytngcnws  nmgnttyatg  gcntaycaya  arccnytnaa  raaywsncar       3180

gaytayacng  argcnytnmg  ngcngcnmgn  garytngcng  cnaayathac  ngcngayytn       3240

mgnaargtnc  cnggnacnga  yccngcntty  gargtnttyc  cntayacnat  hacnaaygtn       3300

ttytaygarc  artayytnac  nathytnccn  garggnytnt  tyatgytnws  nytntgyytn       3360

gtnccnacnt  tygcngtnws  ntgyytnytn  ytnggnytng  ayytnmgnws  nggnytnytn       3420

aayytnytnw  snathgtnat  gathytngtn  gayacngtng  gnttyatggc  nytntgggay       3480

athwsntaya  aygcngtnws  nytnathaay  ytngtnwsng  cngtnggnat  gwsngtngar       3540

ttygtnwsnc  ayathacnmg  nwsnttygcn  athwsnacna  arccnacntg  gytngarmgn       3600

gcnaargarg  cnacnathws  natgggnwsn  gcngtnttyg  cnggngtngc  natgacnaay       3660

ytnccnggna  thytngtnyt  nggnytngcn  aargcncary  tnathcarat  httyttytty       3720

mgnytnaayy  tnytnathac  nytnytnggn  ytnytncayg  gnytngtntt  yytnccngtn       3780

athytnwsnt  aygtnggncc  ngaygtnaay  ccngcnytng  cnytngarca  raarmgngcn       3840

gargargcng  tngcngcngt  natggtngcn  wsntgyccna  aycayccnws  nmgngtnwsn       3900

acngcngaya  ayathtaygt  naaycaywsn  ttygarggnw  snathaargg  ngcnggngcn       3960

athwsnaayt  tyytnccnaa  yaayggnmgn  cartty                                   3996
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(54) Title: NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

(57) Abstract: The present invention provides human, rat and mouse NPCIL1 polypeptides and polynucleotides encoding the polypeptides. Methods for detecting ligands which bind to NPCIL1 and block intestinal cholesterol absorption are provided. Also included is a method of identifying ligands which bind to NPCILI using membranes derived from brush border membrane prepara-tions. Compounds that bind to NPCILI can be used for inhibiting intestinal cholesterol absorption in a subject.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/01469

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53, 33/532, 33534; C12Q 1/60
US CL : 435/7.1,7.93,11; 436/544,545

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
   U.S. : 435/7.1,7.93,11; 436/544,545

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/0132058 A1 (Altmann et al.) 08 July 2004 (08.07.2004), especially paragraph [0011].	1, 7, 8, 10-13
A	US 2004/0161838 A1 (Altmann et al.) 19 August 2004 (19.08.2004), especially paragraph [0011].	1, 7, 8 and 10-13

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2006 (19.01.2006)	1 3 MAR 2006
Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450	Shafiqul Haq
Facsimile No. (571) 273-3201	Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (April 2005)

Continuation of B. FIELDS SEARCHED Item 3:
APS, CAPLUS, STN search, East search.
Search terms : NPC3, NP1C1L1, azetidinone, ezetimibe, label, ligand, agonist, antagonist, detection, identification.